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Award Number: DAMD17-98-1-8279

TITLE: Transcriptional Repression as a Mechanism of BRCA-1 Inactivation

PRINCIPAL INVESTIGATOR: Bernard Futscher, Ph.D.

CONTRACTING ORGANIZATION: University of Arizona
Tucson, Arizona 85722-3308

REPORT DATE: August 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Final (1 Aug 98 - 31 Jul 00)

4. TITLE AND SUBTITLE
Transcriptional Repression as a Mechanism of BRCA-1 Inactivation

5. FUNDING NUMBERS
DAMD17-98-1-8279

6. AUTHOR(S)
Bernard Futscher, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arizona
Tucson, Arizona 85722-3308

E-MAIL:
bfutscher@azcc.arizona.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The basic hypothesis of this project is that aberrant cytosine methylation of the BRCA1 CpG island promoter is a mechanism of BRCA-1 gene inactivation in sporadic human breast cancer. To test this hypothesis, archived frozen human breast cancer specimens were analyzed. Quantitative RT/PCR analysis was used to determine BRCA-1 gene expression levels in breast cancer specimens and cytosine methylation status of the BRCA-1 CpG island promoter was determined using the high resolution bisulfite sequencing technique. We have analyzed 21 axillary node negative breast cancer specimens. Results show a ≥2-fold decrease in BRCA1 mRNA levels in 4 of 21 breast cancer specimens (19%). Of these samples, the three with the lowest levels of BRCA1 expression also showed aberrant methylation of the BRCA1 core promoter (CpG island). Aberrant methylation of BRCA1 core promoter was not detected in any breast cancer samples that had <2-fold decrease in BRCA1 expression. These results suggest that aberrant methylation of the BRCA1 CpG island promoter is associated with down regulation of BRCA1 gene in a subset of human breast cancers, and that BRCA-1 methylation patterns may be useful as a biomarker of disease.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	41
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

The objective of the work in this grant was to determine if 5-methylcytosine-mediated transcriptional repression is a mechanism of inactivation of the tumor suppressor gene BRCA-1 in sporadic human breast cancer. This basic hypothesis was proven correct, and identified a new molecular mechanism by which BRCA1 may become inactivated in sporadic breast cancer. Furthermore, positive results could have a significant impact on breast cancer detection and monitoring. For example, PCR-driven assays that are based on the technical approaches used in this proposal could be used in future translational studies to determine whether the identified changes in cytosine methylation of the BRCA-1 CpG island could serve as a detector of residual disease following bone marrow purging, a detector of microscopic disease in biopsy specimens, or as a prognostic indicator.

Body

Below is a list of the stated tasks.

Task 1: Months 1 - 3: Development of microdissection of normal and tumor tissue from frozen, archived breast cancer specimens. Initial work will be to optimize RNA and DNA isolation from frozen specimens prior to the actual nucleic acid isolation from the breast cancer specimens to be analyzed. Performing control PCR and RT/PCR analysis on the nucleic acids obtained will test quality of the nucleic acids.

Task 2: Months 3 - 12: Microdissection and nucleic acid isolation of normal and breast cancer tissue from archived frozen breast cancer specimens to be analyzed for BRCA1 gene expression and BRCA1 CpG island promoter methylation.

Task 3: Months 9 – 12: Development of a *quantitative* RT/PCR approach for the measurement of BRCA-1 gene expression microdissected specimens.

Task 4: Months 12 - 16: Quantitative RT/PCR analysis of BRCA-1 gene expression in RNA isolated from the 11 paired normal and breast cancer tissues.

Task 5: Months 16 - 23: Cytosine methylation analysis of the BRCA-1 CpG island promoter in DNA isolated from the 11 paired normal and breast cancer tissues.

Task 6: Months 23 - 24: Compilation and analysis of results obtained from the measurement of BRCA-1 gene expression and assessment of cytosine methylation status of the BRCA-1 CpG island promoter in normal and cancerous breast tissue.

We have completed the 6 Tasks proposed in this research grant. The data that reflects these accomplishments is presented in the following text, figures, and appendix. Our results indicate that our hypothesis was correct, and that aberrant cytosine methylation of the BRCA1 CpG island promoter BRCA1 is associated with transcriptional silencing of the BRCA1 tumor suppressor gene in human sporadic breast cancer. These studies will be continued and extended to studies designed to understand the molecular mechanisms by which CpG island methylation participates in gene silencing.

We analyzed the cytosine methylation status of the BRCA1 CpG island promoter in 21 axillary node negative breast cancer specimens by high-resolution bisulfite sequencing [1,5] and BRCA1 expression status was determined by quantitative RT/PCR [2]. The results from these two analyses were blinded from one another until each portion of the study was completed. In Table 1 below, the relative BRCA1 expression of the tumor specimens is shown, as well as the cytosine methylation status of the BRCA1 CpG island of each of the specimens analyzed.

Table 1

BRCA1 mRNA Levels and CpG island methylation status in 21 Breast Cancer Specimens

Tumor specimen	BRCA1 Expression[a]	Methylation status of BRCA1
5	0.02	+
4	0.23	+
1	0.38	+
11	0.50	-
7	0.56	-
19	0.65	-
20	0.71	-
2	0.76	-
12	0.82	-
8	0.83	-
15	0.88	-
10	1.07	-
21	1.08	-
18	1.37	-
24	1.41	-
9	1.46	-
14	1.50	-
23	1.51	-
22	1.92	-
13	1.95	-
16	2.30	-

[a] –BRCA1 expression levels are relative to that of normal human mammary epithelial cells, which were set at a value of 1.0. BRCA1 levels were determined by quantitative RT/PCR and have been previously reported (2).

4 of 21 breast cancer specimens (19%) showed a ≥2-fold decrease in BRCA1 mRNA. Of these samples, the three specimens with the lowest levels of BRCA1 expression also showed aberrant methylation of the BRCA1 core promoter (CpG island). In contrast, aberrant methylation of BRCA1 core promoter was not detected in any breast cancer samples that had <2-fold decrease in BRCA1 expression. The results from these experiments are described below, and in greater depth in our paper in *Carcinogenesis*, which is provided in the Appendix.

Aberrant Methylation of the BRCA1 CpG Island Promoter in Human Breast Cancer

The region of the BRCA1 CpG island that was analyzed contains 30 CpG sites, and is located from –567 to +44 relative to the BRCA1 transcription start site (Figure 1A). The region from –218 to +1 is a bi-directional promoter that regulates the transcription of BRCA1, as well as the NBR2 gene, which lies in a head to head orientation 218 bp upstream of the BRCA1 gene [3]. This short CpG-rich stretch of BRCA1 5' flanking region has previously been shown to contain maximal promoter activity, is unmethylated in normal human mammary epithelial cells,

and is a target region for aberrant methylation associated decreases in BRCA1 expression [4, 5]. The BRCA1 CpG island was amplified from bisulfite-modified DNA obtained from the 21 breast cancer specimens as well as normal controls in a two-step "nested" PCR reaction; the PCR products obtained were subcloned and 20 positive recombinants from each specimen were sequenced. The percent methylation at the 30 CpG sites in the BRCA1 CpG island promoter for

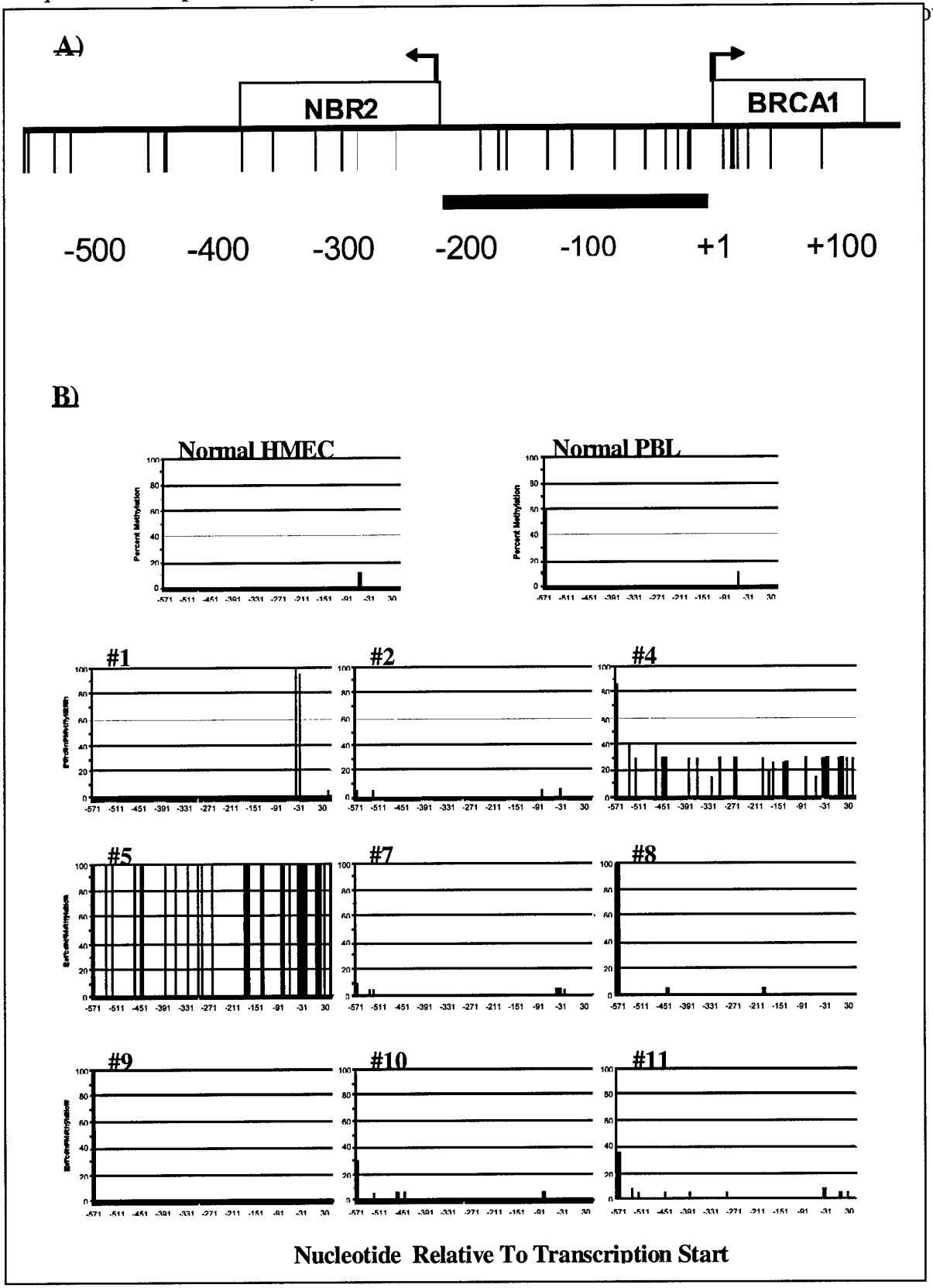
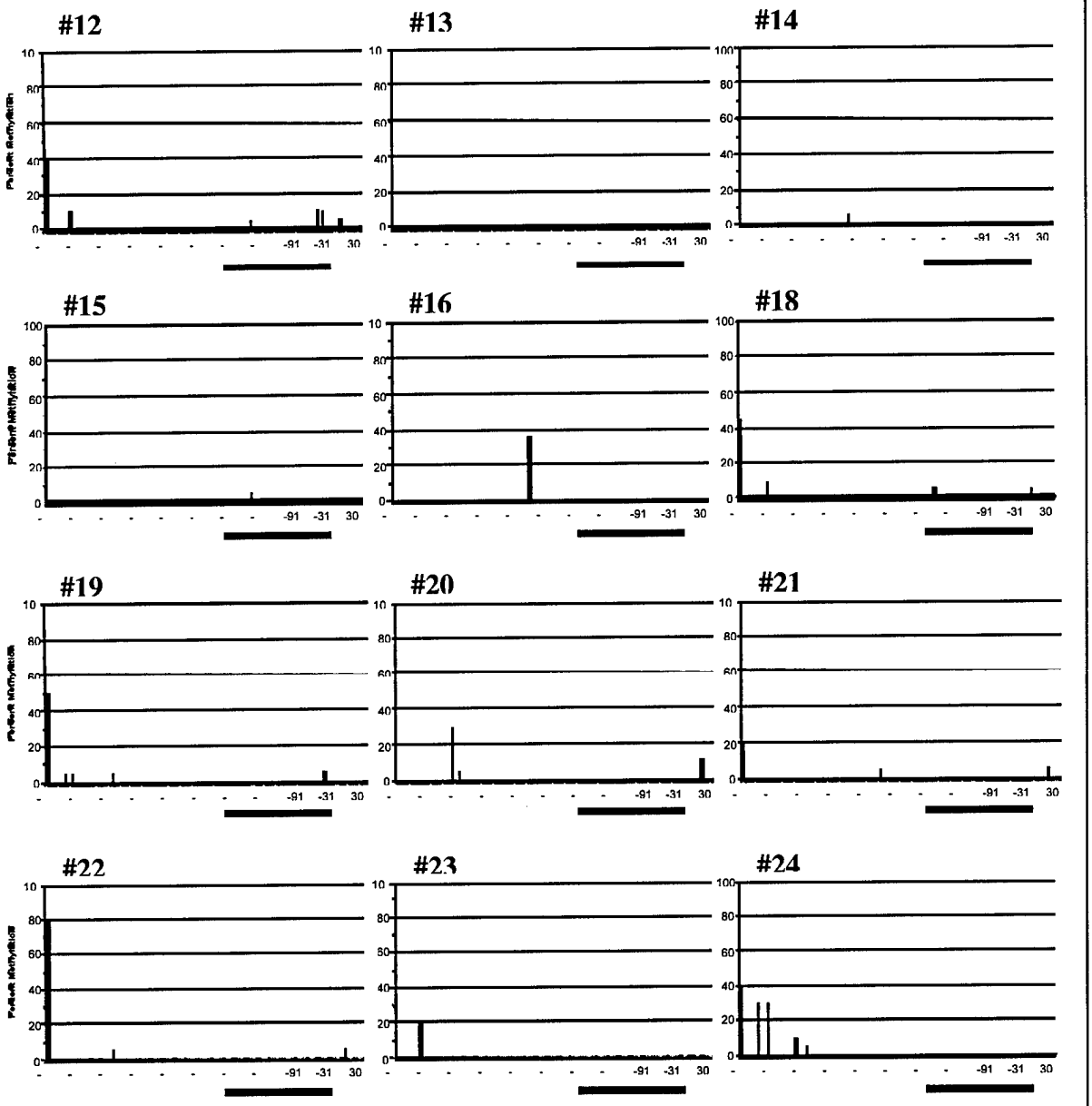


Figure 1B (continued)



A) Schematic representation of the region of the BRCA1 CpG analyzed by bisulfite sequencing. The open rectangles show the position of the first exon for the BRCA1 gene and the NBR2 gene. The bent rectangles show transcription start site and direction. The vertical lines indicate the positions of the 30 CpG sites analyzed and the filled rectangle shows the region of the BRCA1 core promoter. Numbers refer to the nucleotide position relative to BRCA1 transcription start. B) 5-methylcytosine profiles of the BRCA1 CpG island in human breast cancer specimens. One microgram of genomic DNA isolated from the various breast cancer specimens was modified with sodium bisulfite. Briefly, DNA was denatured with 0.3 M NaOH, reacted with 3.6 M sodium bisulfite (pH 5) at 55C for 14 h, desalted by using a Wizard Prep kit (Promega), desulfonated with 0.3 M NaOH, and finally ethanol precipitated in preparation for PCR. The *BRCA1* promoter CpG island was amplified from the bisulfite-modified DNA by two rounds of PCR using nested primers specific to the bisulfite-modified sequence of the *BRCA1* CpG island. The primer sequences and PCR conditions have been previously published (5). The resultant PCR product was cloned into the TA vector pGEM-T-Easy according to the manufacturer's instructions (Promega). Twenty positive recombinants were isolated using a Qiaprep Spin Plasmid Miniprep kit (Qiagen) according to the manufacturer's instructions and sequenced on an ABI automated DNA sequencer. The methylation status of individual CpG sites was determined by comparison of the sequence obtained with the known *BRCA1* sequence. The y-axis represents the percent methylation at the 30 CpG sites in the region amplified; the x-axis represents the nucleotide position relative to the BRCA1 transcription start site. The number of methylated CpGs at a specific site was divided by the number of clones analyzed (n=20 in all cases) to yield the percent methylation for each site.

Three (#1, 4, 5) of the 21 breast cancer specimens analyzed displayed patterns of aberrant cytosine methylation in the 218bp stretch of the BRCA1 core promoter, which contains 11 CpG sites. In contrast the remaining 18 specimens were unmethylated and similar to normal cells. Two of the methylated specimens, #4 and #5, had extensive methylation of all 11 CpG sites in this region. Specimen #5 showed complete methylation in all 20 of the cloned bisulfite PCR products that were sequenced, while #4 showed allelic patterns of cytosine methylation, with ~30% of the DNA molecules showing extensive methylation throughout the promoter; the remainder being unmethylated (see Figure 2 of the*Carcinogenesis* manuscript in the Appendix). This allelic pattern most likely reflects the presence of contaminating normal cells, as well as other possibilities, such as tumor heterogeneity. One of the specimens (#1) showed a unique cytosine methylation pattern with 100% and 95% methylation of two contiguous CpG sites (–37 and –29) within the core promoter region, whereas all other CpG sites remained unmethylated. A search of transcription factor databases of the region surrounding these two CpG sites revealed the presence of a *myb* protein consensus sequence. The *myb* family of DNA-binding proteins is involved in normal breast development and their binding to their DNA consensus site is inhibited by cytosine methylation (6).

Similar to the results obtained for their BRCA1 core promoter regions, specimen #4 and # 5 were also extensively methylated in the 19 CpG sites outside of the core promoter (6 downstream and 13 upstream). In contrast, all other samples were largely unmethylated and similar to normal cells. The two 5' most CpG sites analyzed (-565 and –567) were variably methylated with > 50% methylation of these sites seen in both normal and tumor tissue. These sites may represent a rough 5' boundary of the CpG island, as sites immediately upstream are heavily methylated in both normal and tumor tissue. Although non-CpG cytosine methylation appears to occur in the mammalian genome (7), no evidence for non-CpG cytosine methylation was observed in any of the samples analyzed.

Based on these observations on BRCA1 CpG island methylation in clinical breast cancer specimens, studies were initiated to delineate mechanisms by which CpG island methylation may act to repress gene expression. Using cell line models of the *in vivo* situation, we found that the aberrant methylation of the BRCA1 CpG island was associated with hypoacetylation of both histones H3 and H4 in the BRCA1 CpG island. In turn, these epigenetic changes were associated with a heterochromatinization of the BRCA1 promoter and a loss of BRCA1 gene expression. The details of these experiments are shown in the second manuscript provided in the Appendix, and are in press at *Nucleic Acids Research.*

Key Research Accomplishments

- Identified the BRCA1 CpG island as a target for aberrant methylation in sporadic breast cancer.
- Associated aberrant BRCA1 CpG island methylation with decreased expression of the BRCA1 tumor suppressor gene in sporadic breast cancer.
- Showed that methylation-linked silencing of the BRCA1 gene is associated with histone hypoacetylation and chromatin condensation of the BRCA1 CpG island.

Reportable Outcomes

- A manuscript describing our research results entitled, *"Methylation of the BRCA1 Promoter is Associated with Decreased BRCA1 Expression in Clinical Breast Cancer Specimens"* is in press and will appear in Carcinogenesis in September 2000.

- A manuscript describing our research results entitled, *"Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation, and chromatin remodeling of the BRCA1 5' regulatory region"* is in press and will appear in Nucleic Acids Research in September 2000.

- This breast cancer research grant has supported the graduate training, both living stipend and research supplies, of a doctoral student (Judd C. Rice) who successfully defended his Ph.D dissertation in 6/00. Judd currently is a post-doctoral fellow in the lab of Dr. C. David Allis at the University of Virginia.

- This breast cancer research grant has supported the undergraduate research experience of two separate University of Arizona students, Patrick Maxeiner (98-99) and Nick Holtan (99-00). Patrick Maxeiner went on to become a graduate student in the Pharmacology PhD program at the University of Michigan. Nick Holtan is currently an undergraduate student applying to medical school.

- Results from this IDEA grant are being incorporated into a new NIH RO1 application scheduled to be submitted October 2000.

Conclusions

In conclusion, experimental results obtained in this grant supports our hypothesis that aberrant methylation of the BRCA1 CpG island promoter is associated with decreased levels of BRCA1 gene expression in sporadic human breast cancer at a frequency of ~15%. As the silencing of BRCA1 expression by gene mutation participates in some hereditary forms of breast cancer, our results suggest that BRCA1 silencing by aberrant cytosine methylation may participate in the genesis of sporadic breast cancer in a subset of individuals. *In vitro* studies suggest that methylation of the BRCA1 CpG island is associated with hypoacetylation of the core histones in the BRCA1 promoter and a condensation of the local chromatin resulting in silencing of BRCA1 gene expression. These results indicate that the monitoring of aberrant cytosine methylation of the BRCA1 CpG island in sporadic breast cancer using PCR-based techniques may be useful to detect residual disease following bone marrow purging, or microscopic disease in biopsy specimens, or as a prognostic indicator. Furthermore, these results have encouraged further studies designed to determine if reversal of the aberrantly methylated state of the BRCA1 CpG island may be a target for therapeutic strategies designed to reactivate BRCA1 expression in sporadic human breast cancer.

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Final Report

Publications & Abstracts:

Rice, J.C., Ozcelik, H., Maxeiner, P., Andrulis, I., & Futscher, B.W.: Methylation of the BRCA1 Promoter is Associated with Decreased Expression of BRCA1 Expression in Clinical Breast Cancer Specimens. Carcinogenesis, *in press.*

Rice, J. C. & Futscher B.W.: Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the BRCA1 promoter. Nucleic Acids Research, *in press.*

Bernard W. Futscher, Hilmi Ozcelik, Irene Andrulis, and Judd C. Rice.: BRCA1 promoter methylation in breast cancer patients with low BRCA1 levels. Proceedings from the*Era of Hope* Dept of Defense Breast Cancer Research Program Meeting. pg79, 2000.

Judd C. Rice, Hilmi Ozcelik, Irene Andrulis, Nick Holtan and Bernard W. Futscher: Methylation of the BRCA1 promoter is associated with decreased BRCA1 expression in clinical breast cancer specimens. Proc. AACR. pg658,2000.

Personnel:

Bernard W. Futscher, Ph.D
Judd C. Rice, Ph.D.
Nicholas Holtan
Patrick Maxeiner
R. Ben Isett

Appendices – 2 manuscripts in press are provided on the following pages.

Carcinogenesis vol.21. no.9 pp.000–000, 2000

SHORT COMMUNICATION

Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens

Judd C.Rice[1], Hilmi Ozcelik[2,3], Patrick Maxeiner[6], Irene Andrulis[4,5] and Bernard W.Futscher[1,6,7]

[1]Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721, [2]Centre for Cancer Genetics, Samuel Lunenfeld Research Institute, and Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Tucson, AZ 85724, USA, [3]Department of Laboratory Medicine and Pathobiology, [4]Department of Molecular and Medical Genetics, University of Toronto and [5]Cancer Care Ontario, Toronto, Canada and [6]Bone Marrow Transplant Program, Arizona Cancer Center, Tucson, AZ 85724, USA

[7]To whom correspondence should be addressed
Email: bfutscher@azcc.arizona.edu

Functional inactivation of BRCA1 is an important mechanism involved in breast cancer pathogenesis. Mutation is often responsible for *BRCA1* inactivation in familial breast cancer, but is not responsible for the decreased levels of BRCA1 seen in a subset of sporadic breast cancer patients. To determine if aberrant cytosine methylation of the *BRCA1* promoter is associated with decreased BRCA1 gene expression in human breast cancer, high resolution bisulfite sequence analysis was used to analyze the cytosine methylation status of the *BRCA1* promoter in 21 axillary node negative breast cancer patients with known levels of BRCA1 expression. Aberrant cytosine methylation of the *BRCA1* promoter was detected in three of 21 patient specimens. These three specimens also expressed the lowest levels of BRCA1. Results from this analysis show that aberrant cytosine methylation of the *BRCA1* promoter is directly correlated with decreased levels of BRCA1 expression in human breast cancer, and suggest that epigenetic silencing may be one mechanism of transcriptional inactivation of *BRCA1* in sporadic mammary carcinogenesis.

Mutation of the *BRCA1* tumor suppressor gene is an important contributing factor in hereditary breast cancer; however, *BRCA1* mutations have not been detected in the sporadic form of breast cancer (1,2). Despite the absence of *BRCA1* mutations in sporadic breast cancer, BRCA1 mRNA and protein levels are reduced in a subset of sporadic human breast cancers and breast cancer cell lines (3–9). The decrease in BRCA1 function is associated with the conversion to a malignant phenotype (5,6), which can be reversed by forced re-expression of BRCA1 (10). These data suggest that transcriptional and/or post-transcriptional repression of BRCA1 may participate in the genesis of sporadic breast cancer.

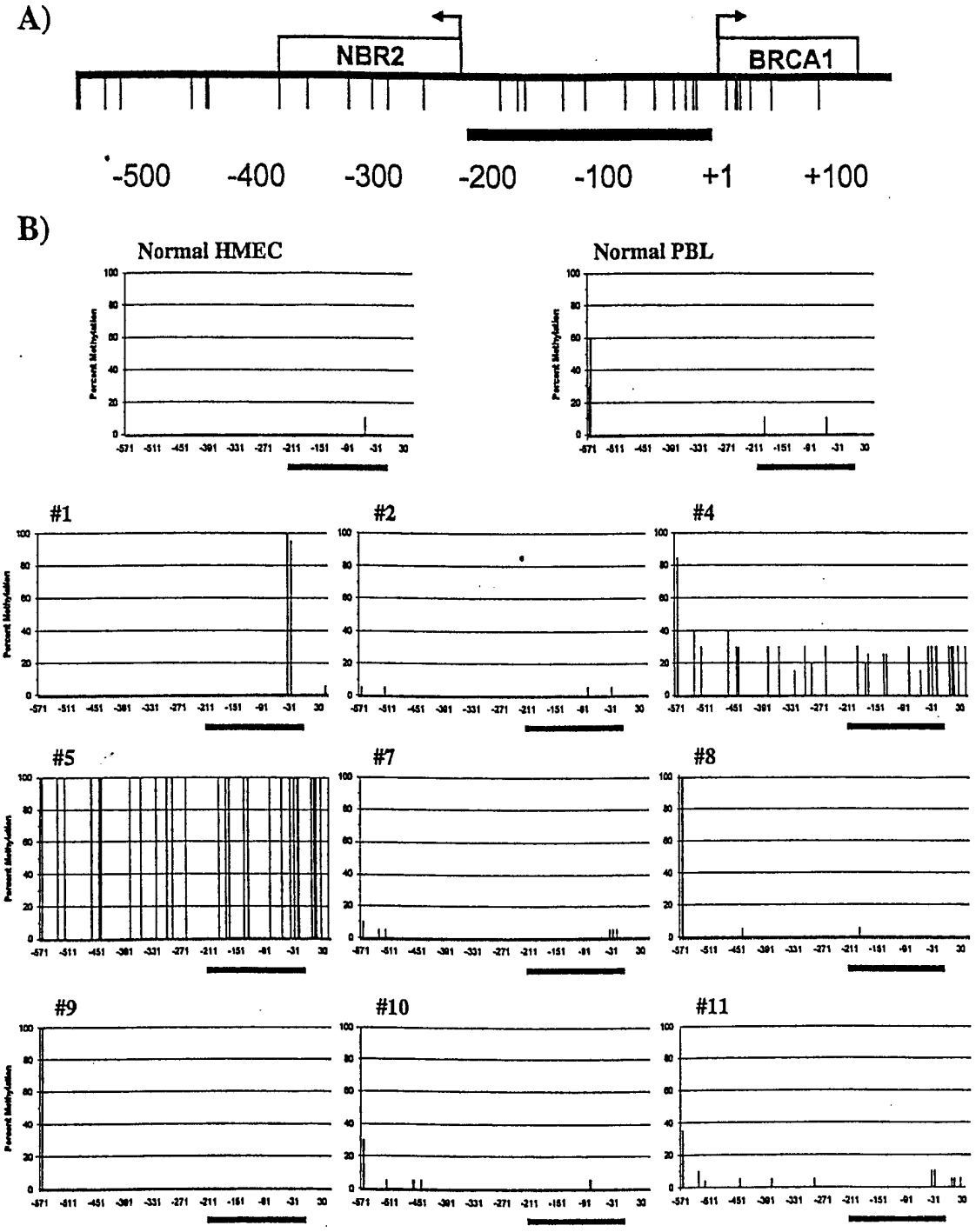
One mechanism of transcriptional repression of tumor suppressor genes in human cancer is aberrant cytosine methylation of their CpG island gene promoters (11). This mechanism has been well documented for a number of genes involved in cancer, and includes Rb, p16, p15, E-cadherin, estrogen receptor and MGMT (12–18). Recently, we have shown that aberrant cytosine methylation of the *BRCA1* CpG island is associated with transcriptional repression in human breast cancer cell lines (4). Other investigators have shown that aberrant methylation of *BRCA1* occurs in human breast cancer specimens; however, BRCA1 expression was not evaluated in these clinical studies (19–22). Together, these data suggest that aberrant cytosine methylation of the *BRCA1* promoter may participate in the transcriptional repression of *BRCA1* gene expression in a subset of sporadic human breast cancers. To further test this hypothesis, the cytosine methylation status of the *BRCA1* promoter in 21 axillary node negative breast cancer specimens with known levels of BRCA1 mRNA (23) were analyzed by high resolution bisulfite sequencing. The breast cancer samples analyzed in the present study are those from which tumor material was still available from the previous study. The results from these two analyses were blinded from one another until the study was completed.

The region of the *BRCA1* CpG island analyzed contains 30 CpG sites, and is located from –567 to +44 relative to the *BRCA1* exon1A transcription start site (Figure 1A). Within this region is a bi-directional core promoter (–218 to +1), that regulates the transcription of *BRCA1*, as well as the *NBR2* gene, which lies in a head-to-head orientation 218 bp from the *BRCA1* gene (24). This short CpG-rich stretch of *BRCA1* 5′ flanking region contains 11 CpG sites and has previously been shown to contain strong promoter activity (25), to be unmethylated in normal human mammary epithelial cells (HMEC) (4) and is a target region for aberrant cytosine methylation in human breast cancer cell lines and tissue specimens (4,19–22).

Genomic DNA (500 ng) isolated from each of the breast cancer specimens was modified with sodium bisulfite using conditions described previously (26). Briefly, DNA was denatured with 0.3 M NaOH, reacted with 3.6 M sodium bisulfite (pH 5) at 55°C for 14 h, desalted by using a Wizard Prep kit (Promega), desulfonated with 0.3 M NaOH, and finally ethanol precipitated in preparation for PCR. The *BRCA1* CpG island was amplified from the bisulfite-modified DNA by two rounds of PCR using nested primers specific to the bisulfite-modified sequence of the *BRCA1* CpG island. These primer sequences and PCR conditions have been published previously (4). The resultant PCR product was cloned into the TA vector pGEM-T-Easy according to the manufacturer's instructions (Promega). For each breast cancer specimen, 20 positive recombinants were isolated using a plasmid miniprep kit (Qiagen), and sequenced on an ABI automated DNA sequencer. Percent methylation of each site was determined by dividing the number of methylated CpGs at a specific site by the total number of clones analyzed (n = 20 in all cases).

From these results, the percent methylation of the 30 CpG sites analyzed in the *BRCA1* CpG island were calculated, and

Abbreviations: HMEC, human mammary epithelial cells.



are shown graphically in Figure 1B. Normal HMEC and normal peripheral blood lymphocytes (PBL) are unmethylated in the 218 bp stretch of the *BRCA1* core promoter with only low-level cytosine methylation detected at a few sporadic sites. Eighteen of the 21 breast cancer specimens were also largely unmethylated and similar to normal cells. In contrast, three of the 21 breast cancer specimens (1, 4 and 5) displayed patterns of aberrant cytosine methylation in the *BRCA1* core promoter region. Two of the breast cancer specimens, 4 and 5, had extensive methylation of all 11 CpG sites in this region. Specimen 5 showed complete methylation at all CpG sites in all 20 of the bisulfite-sequenced PCR products. Specimen 4 showed allelic patterns of cytosine methylation, with ~30% of the DNA molecules showing extensive methylation throughout the promoter with the remainder being largely unmethylated (Figure 2). This allelic pattern most likely reflects many contributing factors, such as the presence of contaminating normal cells in the sample as well as heterogeneity within the tumor. As a representative example for comparison of allelic patterns of cytosine methylation another specimen (specimen 11) is shown in Figure 2. Each row of circles represents the cytosine methylation pattern from an individual clone, and the

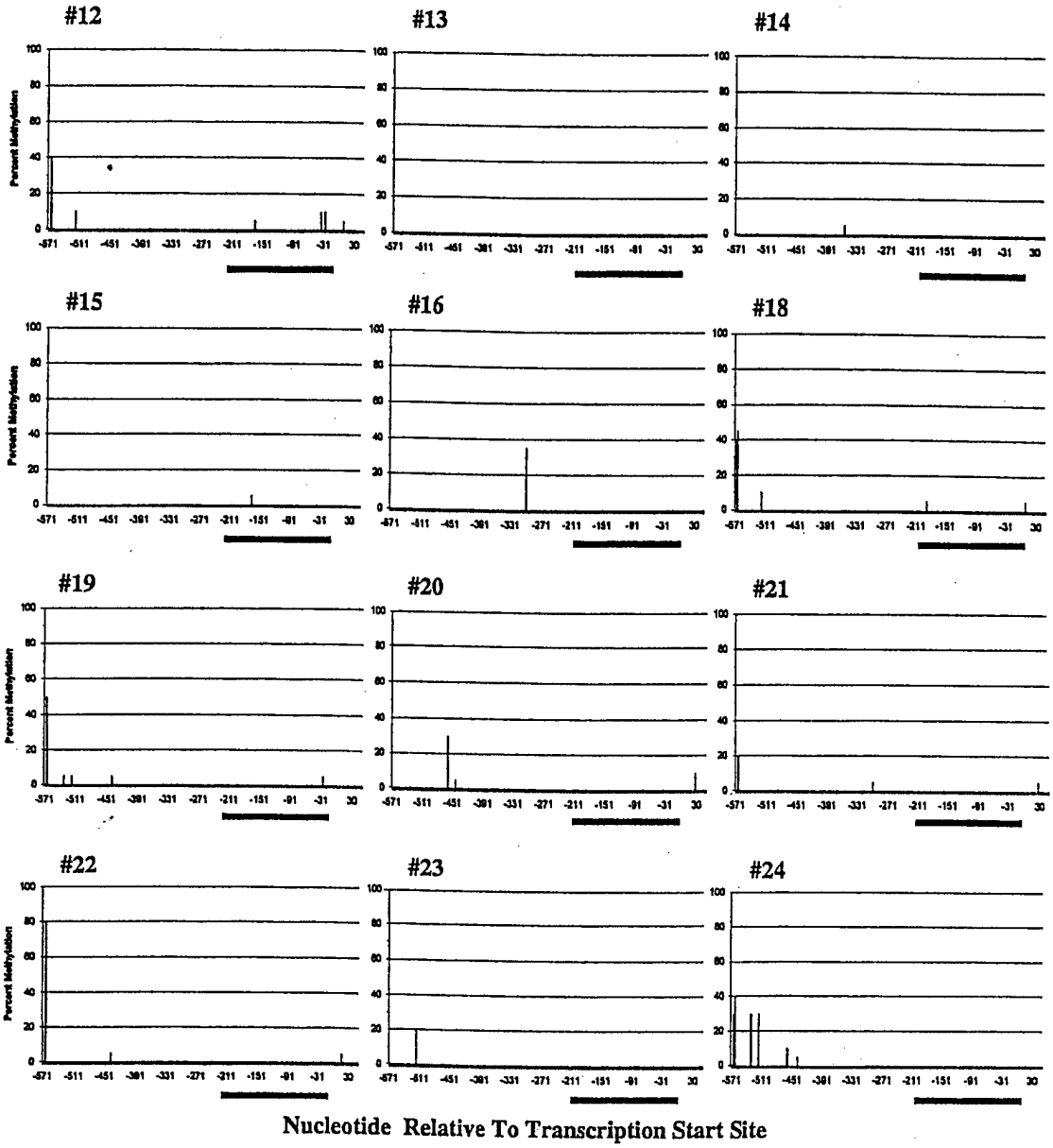


Fig. 1. (A) Schematic representation of the BRCA1 CpG island analyzed by high resolution bisulfite sequencing. The open rectangles show the position of the first exon for the *BRCA1* and *NBR2* genes. The bent arrows show transcription start site and direction. The vertical lines indicate the positions of the 30 CpG sites analyzed, and the heavy horizontal bar shows the region of the *BRCA1* core promoter. Numbers refer to the nucleotide position relative to *BRCA1* transcription start (GenBank accession no. U37574). (B) Cytosine methylation status of the BRCA1 CpG island of 21 human breast cancer specimens. The methylation status of individual CpG sites was determined by comparison of the bisulfite sequence obtained with the known *BRCA1* sequence. The *y*-axis represents the percent methylation at the 30 CpG sites in the region amplified; the *x*-axis represents the nucleotide position relative to the *BRCA1* transcription start site. Percent methylation of each site was determined by dividing the number of methylated CpG sites at a specific site by the total number of clones analyzed (n = 20 in all cases). The line beneath each graph denotes the *BRCA1* core promoter region.

clones shown in Figure 2 were used to calculate the percent methylation of each CpG site for patient specimens 4 and 11 (Figure 1B). In contrast to specimen 4, specimen 11 had low levels of cytosine methylation at a number of CpG sites throughout the entire *BRCA1* CpG island; however, in specimen 11 the sites of cytosine methylation were heterogeneously distributed among the separate alleles, and no extensively methylated alleles were detected.

Breast cancer specimen 1 showed a unique cytosine methylation pattern with 100 and 95% methylation, respectively, of two contiguous CpG sites (–37 and –29) within the core promoter region, whereas all other CpG sites remained unmethylated. Although only two of the 30 total CpG sites analyzed in specimen 1 were methylated, we considered this sufficient to be scored as aberrantly methylated because (i) these sites are unmethylated in normal tissue and (ii) this degree of cytosine methylation would be considered aberrant by other assays that measure cytosine methylation at one or a few sites (e.g. Southern blot). A search of transcription factor databases with the region encompassing these two CpG sites revealed the presence of a *myb* consensus sequence. The *myb* family of DNA-binding proteins is involved in normal breast development and their binding to their DNA consensus sequence has been shown to be inhibited by cytosine methyla-

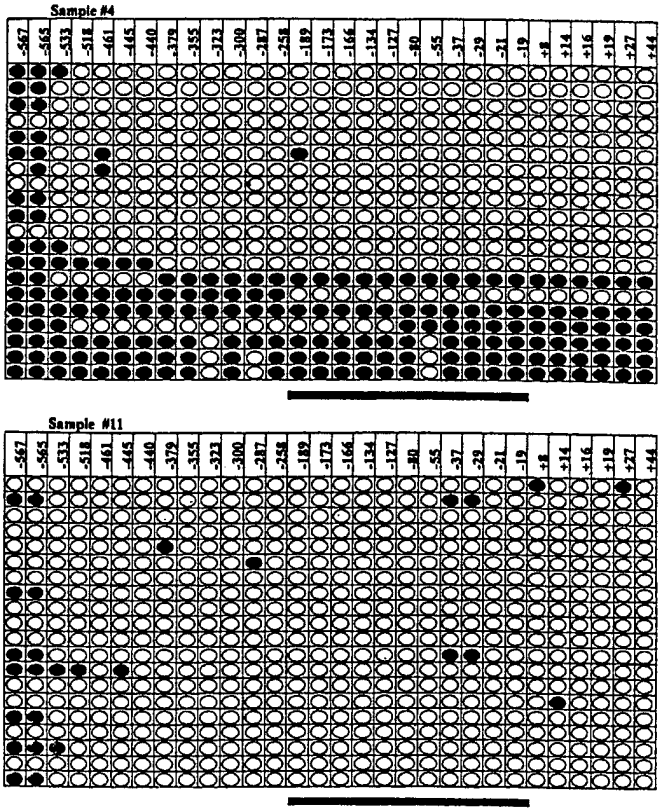


Fig. 2. Allelic patterns of cytosine methylation in the BRCA1 CpG island of two breast cancer specimens. Each row of circles represents the cytosine methylation pattern obtained for individual clones of the *BRCA1* CpG island PCR products obtained from patient specimens 4 and 11. These clones were used to calculate the percent methylation of each CpG site in these specimens (Figure 1B). The position of each CpG site relative to transcription start is shown. Open circles indicate unmethylated CpG sites, filled circles indicate methylated CpG sites. The heavy horizontal bar beneath each graph denotes the *BRCA1* core promoter region.

tion (27–29); however, the functional significance of the pattern of aberrant cytosine methylation observed in specimen 1, if any, remains unknown.

The 19 CpG sites analyzed outside the *BRCA1* core promoter region (11 upstream and six downstream) are completely unmethylated in normal HMEC, whereas PBL showed high methylation of the two most distal 5′ CpG sites analyzed (–565 and –567). These sites may represent a rough 5′ boundary of the CpG island, as sites immediately upstream become increasingly methylated in both normal and tumor tissue (data not shown). Similar to the results obtained for the *BRCA1* core promoter region, specimens 4 and 5 were extensively methylated at the remaining 19 CpG sites. In contrast, all other samples, including specimen 1, were largely unmethylated and similar to normal cells. Although non-CpG cytosine methylation has been reported to exist in the mammalian genome (30), non-CpG cytosine methylation was not detected in any of the normal or tumor tissues analyzed.

Following completion of the cytosine methylation analysis, the level of *BRCA1* gene expression for each of the breast cancer specimens was unblinded. *BRCA1* expression for each breast cancer specimen relative to normal HMEC (23) is shown in Table I, as is their *BRCA1* cytosine methylation status. Four of the 21 breast cancer specimens (1, 4, 5 and 11) expressed levels of BRCA1 of one-half or less compared with normal HMEC. Of these specimens, the three with the lowest relative levels of BRCA1 gene expression also displayed

Table I. BRCA1 mRNA levels and cytosine methylation status in 21 breast cancer specimens

Tumor specimen	BRCA1 expression[a]	Methylation status of BRCA1
5	0.02	+
4	0.23	+
1	0.38	+
11	0.50	–
7	0.56	–
19	0.65	–
20	0.71	–
2	0.76	–
12	0.82	–
8	0.83	–
15	0.88	–
10	1.07	–
21	1.08	–
18	1.37	–
24	1.41	–
9	1.46	–
14	1.50	–
23	1.51	–
22	1.92	–
13	1.95	–
16	2.30	–

[a]BRCA1 expression levels are relative to that of normal human mammary epithelial cells, which were set at a value of 1.0. BRCA1 levels were determined by quantitative RT–PCR and have been reported previously (23).

aberrant cytosine methylation of the *BRCA1* core promoter region (1, 4 and 5). In contrast, aberrant cytosine methylation of *BRCA1* was not detected in any of the 17 specimens that expressed BRCA1 at levels similar to normal cells (i.e. >0.5).

In this study, three of 21 (14%) breast cancer specimens showed aberrant cytosine methylation of the *BRCA1* CpG island. This frequency of aberrant cytosine methylation of the *BRCA1* core promoter in the breast cancer specimens analyzed in this study is consistent with other studies that have analyzed *BRCA1* (3,19–22). Using bisulfite sequencing Mancini *et al.* (22) detected aberrant methylation in two of six breast cancer specimens. Using Southern blot analysis to analyze cytosine methylation of *BRCA1*, Catteau *et al.* detected aberrant *BRCA1* methylation in 10 of 96 breast cancer specimens and Dobrovic and co-workers detected aberrant *BRCA1* methylation in four of 18 patients over the course of two studies, whereas Magdinier *et al.* did not detect aberrant methylation in any of the 37 patients analyzed in their study (3,19–21). A simple compilation of the data from our study and the four studies listed above shows that 19 of 178 (11%) breast cancer specimens displayed aberrant methylation of *BRCA1*. Together, this data probably provides a reasonable estimate of the frequency of aberrant methylation of *BRCA1* in sporadic breast cancer. Furthermore, this estimated frequency of aberrant methylation of the *BRCA1* CpG island in sporadic breast cancer is lower than the frequency of decreased BRCA1 expression in sporadic breast cancer (7–9), indicating that it is likely that multiple mechanisms exist for the inactivation of BRCA1 function in sporadic breast cancer.

The present study extends the earlier studies on *BRCA1* by providing a direct correlation between methylation of the *BRCA1* core promoter and transcriptional repression of the *BRCA1* gene in sporadic human breast cancer, and suggests that 5-methylcytosine-associated inactivation of *BRCA1* may be important in sporadic breast cancer. As forced re-expression

of *BRCA1* inhibits the malignant phenotype, reversal of *BRCA1* promoter methylation may be a potential therapeutic strategy for a subset of sporadic breast cancer patients.

Acknowledgements

We thank Nick Holtan for laboratory assistance and Skip Vaught and his sequencing crew at the Laboratory of Systematics and Evolution, University of Arizona for the automated DNA sequencing. This work is dedicated to the memory of Patrick Maxeiner (1976–2000). This work was supported by Department of Army grant DAMD17-98-1-8279 to B.W.F.

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Received March 21, 2000; revised May 18, 2000; accepted May 24, 2000

Transcriptional repression of *BRCA1* by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the *BRCA1* promoter

ArticleNucleic Acids ResearchNo2817ISSN 0305-1048gkd496http://www.nar.oupjournals.org/content/vol28/issue17/00001September2000

Judd C. Rice[1]

Bernard W. Futscher[1,2,*]

[1]Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721, USA and [2]Bone Marrow Transplant Program, Arizona Cancer Center, Tucson, AZ 85724, USA

Received June 12, 2000

Accepted June 30, 2000

BRCA1 expression is repressed by aberrant cytosine methylation in sporadic breast cancer. We hypothesized that aberrant cytosine methylation of the *BRCA1* promoter was associated with the transcriptionally repressive effects of histone hypoacetylation and chromatin condensation. To address this question, we developed an *in vitro* model of study using normal cells and sporadic breast cancer cells with known levels of *BRCA1* transcript to produce a 1.4 kb 5-methylcytosine map of the *BRCA1* 5′ CpG island. While all cell types were densely methylated upstream of –728 relative to *BRCA1* transcription start, all normal and *BRCA1* expressing cells were non-methylated downstream of –728 suggesting that this region contains the functional *BRCA1* 5′ regulatory region. In contrast, the non-*BRCA1* expressing UACC3199 cells were completely methylated at all 75 CpGs. Chromatin immunoprecipitations showed that the UACC3199 cells were hypoacetylated at both histones H3 and H4 in the *BRCA1* promoter compared to non-methylated *BRCA1* expressing cells. The chromatin of the methylated UACC3199 *BRCA1* promoter was inaccessible to DNA–protein interactions. These data indicate that the epigenetic effects of aberrant cytosine methylation, histone hypoacetylation and chromatin

condensation act together in a discrete region of the *BRCA1* 5′ CpG island to repress *BRCA1* transcription in sporadic breast cancer.

INTRODUCTION

CpG islands are GC-rich regions of DNA that have a higher than expected frequency of CpG dinucleotides. These islands are usually located at the 5′ end of genes and are associated with transcriptional promoters (1). Cytosines of CpG islands are non-methylated in normal tissues regardless of transcription status, whereas aberrant cytosine methylation of the 5′ CpG islands of genes is commonly associated with their transcriptional repression (2).

The aberrant cytosine methylation of CpG islands is associated with the alteration of chromatin structure to a protein-inaccessible state, which appears to participate in the transcriptional repression of the associated gene (3–5). A protein-inaccessible chromatin structure is also directly linked to the acetylation status of core histones in the nucleosomes of gene promoters (6,7). Hypoacetylated histones are associated with transcriptionally inert regions of heterochromatin, whereas acetylated histones are associated with transcriptionally active regions of euchromatin (8–10). Recent reports have identified a mechanistic pathway of epigenetic silencing by cytosine methylation, histone hypoacetylation and chromatin condensation suggesting that these mechanisms act together to inactivate gene transcription (11,12).

In this study we investigated the mechanisms of epigenetic silencing of the breast cancer susceptibility gene *BRCA1* in sporadic breast cancer. *BRCA1* is a tumor suppressor gene whose expression is repressed in a large portion of sporadic breast cancer patients and is associated with a malignant phenotype (13–15). Recent reports indicate that aberrant cytosine methylation of *BRCA1* occurred in two of six and two of seven sporadic breast cancer specimens, respectively (16,17). In addition to these studies, we used high resolution bisulfite sequencing to identify aberrant cytosine methylation of the *BRCA1* 5′ CpG island in three of 21 sporadic breast cancer specimens. These three specimens also expressed the lowest levels of *BRCA1* transcript by RT–PCR analysis (~~*Carcinogenesis*, in press~~). We hypothesized that the aberrant cytosine methylation of the *BRCA1* promoter is associated with histone hypoacetylation, chromatin condensation and transcriptional repression of *BRCA1* in sporadic breast cancer.

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To test this hypothesis, we developed an *in vitro* model of study using normal cells and sporadic breast cancer cells with known levels of *BRCA1* transcript to produce a 1.4 kb 5-methylcytosine map of the *BRCA1* 5′ CpG island. High resolution bisulfite sequence analysis showed that the non-methylated CpG island domain extends downstream of –728 relative to

transcription start in normal and *BRCA1* expressing cells. In contrast, the region upstream of −728, although still CpG rich, was methylated in both normal cells and breast cancer cell lines. The non-methylated domain contains maximal *BRCA1* promoter activity and is the target region for aberrant cytosine methylation in breast cancer cells (17–20). We identified one *BRCA1*-negative sporadic breast cancer cell line that was aberrantly methylated at all 75 CpG dinucleotides analyzed. Chromatin immunoprecipitation assays revealed that the aberrantly methylated *BRCA1* promoter of the *BRCA1*-negative breast cancer cells is associated with hypoacetylated histones H3 and H4 compared to the non-methylated *BRCA1* promoter of normal and tumorigenic breast cells that express *BRCA1*. In addition, the aberrant cytosine methylation and histone hypoacetylation of the *BRCA1* promoter coincides with a protein-inaccessible chromatin structure and transcriptional repression of *BRCA1*. These data indicate that the epigenetic effects of aberrant cytosine methylation, histone hypoacetylation and chromatin condensation act together in the discrete region of the *BRCA1* promoter to repress *BRCA1* transcription in sporadic breast cancer.

MATERIALS AND METHODS

Cell culture

MCF7 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Gibco BRL, Grand Island, NY), 100 U/ml penicillin (Gibco BRL) and 1% glutamate (Gibco BRL) in a 5% CO_2 atmosphere at 37°C. UACC3199 is an early passage sporadic breast cancer cell line derived from an infiltrating ductal carcinoma isolated from axillary lymph nodes. In this study, UACC3199 was passaged not more than 20 times and maintains the original genotype and phenotype of the primary tumor (21,22). Normal human mammary epithelial cells (HMEC) were purchased from Clonetics (San Diego, CA) and grown according to the manufacturer's instructions. The peripheral blood lymphocytes (PBL) and human foreskin fibroblasts (HFF) are primary cells.

High resolution bisulfite sequencing of the BRCA1 5′ region

Genomic DNA was modified with sodium bisulfite as previously described (19). A 1.4 kb sequence of the *BRCA1* 5′ flanking region was amplified in two separate nested PCR reactions. A 656 bp nested PCR product from −591 to +66 relative to the *BRCA1* transcription start site was amplified from the bisulfite-modified DNA with bisulfite specific primers derived from the reported *BRCA1* sequence (GenBank accession no. U37574). The primer sets used were as follows:

primer 1 (nt 895–916), 5′-GGGGTTGGATGGGAATTGTAG-3′;
primer 2 (nt 1688–1792), 5′-CTCTACTACCTTTACCCAAAAACA-3′;
primer 3 (nt 989–1013), 5′-GTTTATAATTGTTGATAAGTATAAG-3′;
primer 4 (nt 1626–1646), 5′-AAAACCCCACAACCTATCCC-3′.

An 802 bp fragment of the *BRCA1* upstream region from –1369 to –567 was also amplified from the bisulfite-modified DNA by nested PCR using the following primer sets:
primer 1 (nt 177–200), 5′-TTAGTTTAGAGAGGGGTTTTTATA-3′;
primer 2 (nt 1094–1119), 5′-CCACAATATTCCTTAAAAACTATAAT-3′;
primer 3 (nt 211–232), 5′-GGGTTGAAGGGTTTTTTTAGTA-3′;
primer 4 (nt 989–1013), 5′-CTTATACTTATCAACAATTATAAAC-3′.
Primers 1 and 2 were used in the first round of amplification and primers 3 and 4 were used in the second round of amplification under the following conditions: 95°C for 1 min followed by 35 cycles of 92°C for 1 min, 56°C for 3 min, 72°C for 1 min; ending with a final extension of 72°C for 5 min and a quick chill to 4°C. The resultant PCR products were cloned into pGEM-T-Easy TA (Promega, Madison, WI) according to the manufacturer's instructions. Positive recombinants were isolated using the Qiaprep Spin Plasmid Miniprep Kit (Qiagen, ?? **location of supplier** ??) and sequenced on an ABI automated DNA sequencer. Ten recombinants from each cell type were analyzed for the *BRCA1* regulatory region. For the *BRCA1* upstream region, ten recombinants were analyzed for PBL and HFF, eight recombinants for HMEC, and six recombinants for MCF7 and UACC3199. All non-methylated cytosines were successfully converted in the bisulfite reaction in all of the recombinants analyzed (Fig. S2).

VALENCIA, CA

Acetyl-histone H3 and H4 chromatin immunoprecipitations and PCR amplification of the BRCA1 and GAPDH promoters

Chromatin immunoprecipitations using the acetyl-histone H3 and H4 antibodies were performed according to the manufacturer's instructions (Upstate Biotech, Lake Placid, NY). Cells were rinsed in 1× PBS and treated with 1% formaldehyde for 10 min at 37°C to form DNA–protein cross-links. The cells were rinsed in ice cold 1× PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A), scraped and collected by centrifugation at 4°C. Cells were resuspended in an SDS lysis buffer containing protease inhibitors and incubated on ice for 10 min. The DNA–protein complexes were sonicated to lengths between 200 and 1000 bp as determined by gel electrophoresis (Fig. S3), isolated by centrifugation, and diluted with buffer containing protease inhibitors. The sample was divided equally into thirds: +Ab, –Ab, and control sample. The +Ab and –Ab samples were pre-cleared with a Salmon Sperm DNA/Protein A Agarose Slurry. Following pre-clearing,

the +Ab sample was exposed to the acetyl-histone H3 or H4 antibody and all of the samples were incubated overnight at 4°C with rotation. The chromatin–antibody complexes were collected using the Salmon Sperm DNA/Protein A Agarose Slurry and then sequentially washed with the manufacturer's low salt buffer, high salt buffer, LiCl buffer, and twice with Tris–EDTA. The chromatin–antibody complexes were eluted and the DNA–protein cross-links were reversed with 5 M NaCl at 65°C for 4 h. All samples were treated with proteinase K and the acetyl-histone H3 or H4 enriched fraction of genomic DNA was recovered by phenol/chloroform extractions and ethanol precipitations and quantitated using pico green (Molecular Probes, Eugene, OR). PCR amplification of the *BRCA1* promoter (GenBank accession no. U37574) was performed using the following primers:
primer 1 (nt 1349 nt 1369), 5′-GGCAGGCACTTTATGGCAAAC-3′,
primer 2 (nt 1757 nt 1778), 5′-TTCGGAAATCCACTCTCCCACG-3′.
PCR amplification of the *GAPDH* promoter (GenBank accession no. J04038) was performed using the following primers:
primer 1 (nt 827 nt 851), 5′-TAGTGTCCTGCTGCCCACAGTCCAG-3′,
primer 2 (nt 1168 nt 1187), 5′-GGCGACGCAAAAGAAGATGC-3′.
Both PCRs were performed under the following conditions: 95°C for 4 min followed by 40 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 2 min and ending with a final extension of 72°C for 5 min. PCR products were size fractionated on a 3% TBE agarose gel, stained with ethidium bromide, and visualized on the Eagle Eye II Still Video System (Stratagene, La Jolla, CA).

Chromatin accessibility assays of the BRCA1 5′ region

Chromatin accessibility assays were performed as previously described (5) with minor modifications. Twenty million cells were washed twice with 1× PBS, gently scraped and collected by centrifugation. Nuclei were extracted by resuspension of cells in ice cold 1× RSB (10 mM Tris–HCl pH 8, 3 mM $MgCl_2$, 10 mM NaCl, 0.05% NP-40). The nuclei were collected by centrifugation, resuspended in appropriate 1× restriction endonuclease buffer, and divided into four aliquots of 200 µl/aliquot. Zero, 25, 75, or 225 U of either *Eco*RI or *Sst*I (Gibco BRL) was added to the nuclei and incubated at 37°C for 15 min. Genomic DNA was isolated using the QIAamp Tissue Kit (Qiagen) and 7.5 µg of this DNA was digested with 25 U of *Bam*HI (Gibco BRL). Following phenol/chloroform extractions, the DNA was size-fractionated on a 1% TBE agarose gel and capillary transferred onto a 0.45 µm pore size Nytran Plus membrane (Schleicher and Schuell, Keene, NH). The membranes were hybridized with a probe generated from –564 to –204 of the *BRCA1* 5′ region and ^{32}P-labeled using the random primer method. Membranes were washed once in 2× SSC–0.5% SDS for

30 min at room temperature followed by two washes at 62°C for 30 min in a 0.1% SSC–0.5% SDS solution. Results were visualized by autoradiography.

RESULTS

5-methylcytosine map of the BRCA1 5′ region

We developed an *in vitro* model of study using normal cells and sporadic breast cancer cells with known levels of *BRCA1* transcript to produce a 1.4 kb 5-methylcytosine map of the *BRCA1* 5′ CpG island. The cell types used to generate this 5-methylcytosine map were the *BRCA1*-positive normal HMEC, *BRCA1*-negative normal human PBL, normal HFF and the *BRCA1*-positive MCF7 and *BRCA1*-negative UACC3199 human sporadic breast cancer cell lines (19). The presence of 5-methylcytosine in the 1.4 kb sequence of the *BRCA1* 5′ region was determined by bisulfite sequencing of two separate PCR amplicons. The first nested PCR amplification contained 30 CpG dinucleotides and extended from –591 to +66 relative to the *BRCA1* transcription start site. The second nested PCR amplification was the *BRCA1* 5′ upstream region which contained 45 CpG dinucleotides and extended from –1369 to –567. Clones from each cell type were analyzed to create a high resolution 5-methylcytosine map of the *BRCA1* 5′ region as illustrated in Figure 1. The sequence information obtained for each cell type was compared to the known *BRCA1* sequence to determine the frequency of 5-methylcytosine for each CpG dinucleotide. (Fig. 1)

The five cell types analyzed were densely methylated in the 5′ region upstream of –728. In contrast, 5-methylcytosine was absent in all normal cells and the *BRCA1*-positive MCF7 cells downstream of –728 suggesting that this non-methylated domain is the functional 5′ regulatory region of the *BRCA1* CpG island. Unlike the other cell types, the *BRCA1*-negative UACC3199 cells were completely methylated downstream of –728. The aberrant cytosine methylation of the *BRCA1* promoter in UACC3199 is associated with an observed 10-fold decrease in *BRCA1* transcript compared to HMEC (19). These results indicate that aberrant cytosine methylation of the *BRCA1* promoter is associated with transcriptional repression of *BRCA1*.

Aberrant cytosine methylation of the *BRCA1* promoter is associated with histone H3 and H4 hypoacetylation

To determine if aberrant cytosine methylation of the *BRCA1* promoter was associated with histone hypoacetylation, chromatin immunoprecipitations of UACC3199, MCF7 and HMEC were performed using acetyl-histone H3 and H4 antibodies. Following chromatin immunoprecipitations, the acetyl-histone H3 and H4 enriched fraction of genomic DNA was

purified and isolated. The enriched DNA was analyzed by PCR for the presence of the *BRCA1* promoter (from –232 to +198), as well as the constitutively active *GAPDH* promoter (from –311 to +50).

Figure 2A shows the results of one of the acetyl-histone H3 immunoprecipitation experiments. The acetyl-histone H3 enriched genomic DNA from the methylated *BRCA1*-negative UACC3199 cells failed to amplify the 429 bp *BRCA1* PCR product compared to the non-methylated *BRCA1*-positive HMEC and MCF7 cells as seen in the +H3 Ab lanes. The *BRCA1* PCR product was not amplified in the no acetyl-histone H3 antibody control (–H3 Ab lanes), but was amplified in the genomic DNA positive control in all cell types analyzed. As expected, each cell line had acetylated histone H3 associated with the constitutively expressed *GAPDH* CpG island promoter.

Similar results were obtained from the acetyl-histone H4 immunoprecipitation experiments. Figure 2B shows that the acetyl-histone H4 enriched genomic DNA from the methylated *BRCA1*-negative UACC3199 cells failed to amplify the 429 bp *BRCA1* PCR product compared to the non-methylated *BRCA1*-positive HMEC and MCF7 cells as seen in the +H4 Ab lanes. The *BRCA1* PCR product was not amplified in the no acetyl-histone H4 antibody control (–H4 Ab lanes), but was amplified in the genomic DNA positive control in all cell types analyzed. Each cell line had acetylated histone H4 associated with the constitutively expressed *GAPDH* CpG island promoter.

The appearance of faint bands in the –Ab lanes of all the *BRCA1* PCR samples and different band intensities of the positive control samples underscores that, based on the limitations of the assay, these experiments are purely qualitative in nature and that degrees of (hypo)acetylation of each histone cannot be quantitated. However, Figure 2 does show that the methylated, *BRCA1*-negative UACC3199 cells are hypoacetylated at histones H3 and H4 compared to the non-methylated, *BRCA1*-positive HMEC and MCF7 cells. These data indicate that active transcription of *BRCA1* and *GAPDH* coincides with a non-methylated and histone acetylated promoter. In contrast, the aberrant cytosine methylation and histone hypoacetylation of the UACC3199 *BRCA1* promoter is associated with the observed transcriptional repression of *BRCA1*.

Aberrant cytosine methylation and histone hypoacetylation prevent accessibility of proteins to the *BRCA1* promoter

Chromatin accessibility assays were performed to determine if the aberrant cytosine methylation and histone H3 and H4 hypoacetylation of the *BRCA1* promoter were associated with the remodeling of chromatin to a transcriptionally repressive state. We investigated two regions of the *BRCA1* 5′ region for protein accessibility: the methylated upstream region and

the *BRCA1* promoter. Intact nuclei were isolated from the non-methylated, histone acetylated, *BRCA1*-positive HMEC and MCF7 cells, and the methylated, histone hypoacetylated, *BRCA1*-negative UACC3199 cells. The nuclei were subjected to an *in vivo* restriction endonuclease digestion by *Eco*RI or *Sst*I. Following the *in vivo* digestion and isolation of genomic DNA, an *in vitro* restriction endonuclease digestion was performed with *Bam*HI to release DNA fragments of predictable sizes for Southern analysis.

The *BRCA1* 5′ region has *Eco*RI restriction sites in both the methylated 5′ region at –1640 and in the *BRCA1* promoter at –204 (Fig. 3A). Nuclei from HMEC, MCF7 and UACC3199 were exposed to 0, 25, 75 or 225U of *Eco*RI for 15 min. Following isolation of genomic DNA, the samples were digested *in vitro* using *Bam*HI, which cleaves at –1844 and +756 to yield a 2600 bp fragment. A radiolabeled probe was designed between the *Eco*RI cut sites such that a maximum of four possible products could be visualized by Southern analysis, as illustrated in Figure 3A. Inaccessibility of *Eco*RI at both restriction sites yields a 2600 bp fragment, which is represented by the no *Eco*RI control located in the first lane of each cell type. Accessibility of *Eco*RI in the upstream region only would result in a 2396 bp fragment, whereas accessibility of *Eco*RI in the promoter only would result in a 1641 bp fragment. Accessibility at both *Eco*RI sites would yield a 1437 bp fragment, as represented by the *in vitro* positive control shown in the fifth lane of each cell type. (Fig. 3)

Figure 3B shows the results of one of the *Eco*RI chromatin accessibility experiments. The *Eco*RI site in the *BRCA1* promoter was accessible to enzymatic cleavage in the non-methylated, histone acetylated, *BRCA1*-positive MCF7 and HMEC cells with as little as 25 U of *Eco*RI as illustrated by the appearance of a 1641 bp fragment in the second lane of these samples. In contrast, the *BRCA1* promoter was inaccessible to *Eco*RI cleavage in the methylated, histone hypoacetylated, *BRCA1*-negative UACC3199 cells at all concentrations of *Eco*RI as demonstrated by the absence of the 1641 bp fragment or 1437 bp fragment in the second, third and forth lanes. The *Eco*RI site located at –1640 in the methylated upstream region flanking the *BRCA1* 5′ regulatory region was inaccessible to all concentrations of *Eco*RI in UACC3199, MCF7 and HMEC, as evidenced by the lack of either the 2396 or the 1437 bp fragment.

A similar chromatin accessibility assay was performed using the *Sst*I restriction endonuclease as the *in vivo* restriction endonuclease (Fig. 4). The *Sst*I restriction sites reside at –1723 in the methylated upstream region and at +26 in exon 1a. Using the same radiolabeled probe as the *Eco*RI experiments, Southern analysis yields four possible products as illustrated in Figure 4A. Inaccessibility of *Sst*I at both restriction sites yields a 2600 bp fragment, which is represented by the no *Sst*I control located in the first lane of each cell type **[?? relates to Figure 4A or B ??]**. Accessibility of *Sst*I in the methylated upstream region (Fig. 4)

FIGURE 4B

only would result in a 2479 bp fragment, whereas, accessibility of *Sst*I in exon 1a only would result in a 1870 bp fragment. Accessibility at both *Sst*I sites would yield a 1749 bp fragment, as represented by the *in vitro* positive control shown in the fifth lane of each cell type.

Figure 4B shows the results of one of the *Sst*I chromatin accessibility experiments. The *Sst*I site located near the *BRCA1* transcription start site was accessible to as little as 25 U of *Sst*I in the non-methylated, histone acetylated, *BRCA1*-positive MCF7 and HMEC cells as illustrated by the appearance of the 1870 bp fragment in the second lane of each of these samples. In contrast, the methylated, histone hypoacetylated, *BRCA1*-negative UACC3199 cells were inaccessible to all concentrations of *Sst*I as demonstrated by the absence of the 1870 bp fragment or 1749 bp fragment in the second, third and fourth lanes. The *Sst*I site located at –1723 in the methylated upstream region flanking the *BRCA1* 5′ regulatory region was inaccessible to all concentrations of *Sst*I in UACC3199, MCF7 and HMEC, as evidenced by the lack of either the 2479 bp fragment or the 1749 bp fragment.

These data indicate that chromatin condensation of the *BRCA1* promoter coincides with the aberrant cytosine methylation and histone hypoacetylation of this discrete region of DNA, and the transcriptional inactivation of *BRCA1*. A summary of these results is shown in Figure 5. (Fig. 5)

DISCUSSION

In this study we used an *in vitro* sporadic breast cancer cell line, UACC3199, with methylation-associated *BRCA1* repression to analyze histone H3 and H4 acetylation status as well as chromatin structure of the *BRCA1* promoter. Our findings show that aberrant cytosine methylation of the *BRCA1* promoter coincides with the transcriptionally repressive effects of histone hypoacetylation and chromatin condensation. The aberrant cytosine methylation of the *BRCA1* promoter observed in UACC3199 is a selective event as the 5′ CpG islands of the *p15* and deoxycytosine kinase genes were found to be non-methylated (Fig. S1). The methylated *BRCA1* promoter of the *BRCA1*-negative UACC3199 cells contain hypoacetylated histones H3 and H4 compared to the non-methylated, *BRCA1*-positive HMEC and MCF7 cells indicating that histone hypoacetylation occurs only in the aberrantly methylated *BRCA1* promoter. In contrast to the *BRCA1*-positive HMEC and MCF7 cells, the methylated and hypoacetylated *BRCA1* promoter of UACC3199 contains condensed chromatin that coincides with the observed transcriptional repression of *BRCA1*.

Taken together, our data indicates that aberrant cytosine methylation, histone hypoacetylation and chromatin condensation act together in the discrete region of the *BRCA1* promoter to inactivate *BRCA1* transcription in sporadic breast cancer. This is the first study

to show the direct association of these three epigenetic events in the inactivation of a human tumor suppressor gene. Our findings are consistent with recent reports of the imprinted human fragile X mental retardation gene, *FMR1*. The *FMR1* gene is inactivated in fragile X cells by cytosine methylation, hypoacetylation of histones H3 and H4 and chromatin condensation in its 5′ regulatory region compared to normal cells (22,23). Similarly, in an *in vitro* system, a methylated herpes simplex virus thymidine kinase reporter is transcriptionally repressed in stably transfected L-cells and is associated with hypoacetylated histone H4 and DNase I insensitivity (24). These studies directly indicate that cytosine methylation, histone hypoacetylation and chromatin condensation act together in mammalian gene promoters to suppress transcription.

In contrast to the non-methylated *BRCA1* promoter, the region upstream of –728 is densely methylated in all normal cell types analyzed regardless of *BRCA1* expression. In addition, the chromatin accessibility assays show that this methylated region is inaccessible to *in vivo* endonuclease digestion. Although we attempted to determine the acetylation status of the 5′ methylated region, the presence of a repetitive element upstream of –1273 blunted our efforts to successfully analyze this region. These data indicate that the methylated region upstream of –728 is inaccessible to DNA–protein interactions and suggests that the non-methylated and accessible region downstream of –728 represents the functional 5′ regulatory region of the *BRCA1* CpG island. This conclusion is reinforced by *BRCA1* promoter studies which show that reporter constructs lacking the region upstream of –728 have significantly higher promoter activities compared to reporter constructs that contain the region upstream of –728 (18,19).

The 5-methylcytosine pattern of the *BRCA1* CpG island observed in the normal cells is typical of other human tumor suppressor gene CpG islands such as E-cadherin and VHL (25). While the 5′ regulatory region of these genes are non-methylated in normal cells, the upstream region contains a methylated repetitive element. Recent studies suggest that methylated repetitive elements may function as *cis*-acting '*de novo* methylation centers' (26,27). In cultured human fibroblasts overexpressing DNA methyltransferase, methylation spread from the methylated repetitive element into the 5′ regulatory regions of both the E-cadherin and VHL genes (25). The mouse *aprt* gene is protected from its 5′ *de novo* methylation center by the presence of an Sp1 site located in the 5′ regulatory region (28). Deletion of this Sp1 site results in the aberrant cytosine methylation of the *aprt* regulatory region and transcriptional repression of *aprt* (29). It is possible that, like *aprt*, the *BRCA1* 5′ regulatory region is protected from aberrant cytosine methylation by DNA-binding proteins located in the region around –728. It is interesting to note that there is an Sp1 consensus sequence located at –687 between the last methylated CpG of the upstream methylated

domain and the first non-methylated CpG dinucleotide of the non-methylated regulatory region.

Based on these results and the current scientific literature, we propose the following temporal sequence of epigenetic repression of *BRCA1* in sporadic breast cancer. The aberrant cytosine methylation of the *BRCA1* 5′ regulatory region is, most likely, the first epigenetic event. Previous studies have shown that a methylated reporter construct transfected into mammalian cells was able to transcribe the reporter gene for 8 h (31,32). After 8 h, however, the methylated construct became transcriptionally inert which coincided with alterations in the nucleosomal array and an inability of RNA polymerase to bind the regulatory region. These data indicate that methylation of the regulatory region is not directly responsible for transcriptional repression, rather, methylation leads to a transcriptionally repressive chromatin state.

The aberrant cytosine methylation of the *BRCA1* 5′ regulatory region may be followed by the binding of methylation specific methyl binding proteins (33–35). One of these methyl binding proteins, MeCP2, has been shown to associate with a transcriptional repressor complex that includes histone deacetylases (11,12). We speculate that the methylated *BRCA1* 5′ regulatory region recruits MeCP2, or an analogous methyl binding protein, and a repressor complex that is capable of deacetylating histones H3 and H4, as observed. In turn, the deacetylation of histones, probably in concert with other chromatin remodeling proteins, results in the observed chromatin condensation and transcriptional repression of *BRCA1*.

This study shows that aberrant cytosine methylation, histone hypoacetylation, and chromatin condensation act together in a discrete region of the *BRCA1* 5′ CpG island to inactivate *BRCA1* transcription. Our data suggests that the aberrant cytosine methylation observed in other human tumor suppressor gene CpG island promoters coincide with alterations in the composition and structure of the associated chromatin to a transcriptionally repressive state. These epigenetic alterations may reflect a common set of events necessary for the inappropriate transcriptional inactivation of human tumor suppressor genes and the progression of cancer.

SUPPLEMENTARY MATERIAL

Supplementary Material available at NAR Online.

ACKNOWLEDGEMENTS

We thank Elliot Epner and David Bearss for critical review of the manuscript, Kathy Massey-Brown of the Arizona Cancer Center Cell Culture Core Facility for her assistance

with cell culture, Skip Vaught and his sequencing crew at the Laboratory of Systematics and Evolution, University of Arizona, for the automated DNA sequencing, Nick Holtan for his assistance with the upstream 5-methylcytosine mapping and Jonathan Dodge for his assistance with the 5-methylcytosine mapping of *p15* and *dCK*. This work was supported by NCI 65662 and DAMD 17-98-18279 to B.W.F. and 3P30CA23074-19 to the Arizona Cancer Center.

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*To whom correspondence should be addressed at: Arizona Cancer Center, Tucson, AZ 85724, USA. Tel: +1 520 626 4646; Fax: +1 520 626 2413; Email: bfutscher@azcc.arizona.edu **[?? Author- please confirm contact details are correct as fax no. differs to that on manuscript submittal form ??]***

FAX +1 520-626-2415

18 RICE, J.C., OZCELIK, H., MAXEINER, P., ANDROULIS, I., and FUTSCHER, B.W. (2000) CARCINOGENESIS, 21(9), in press.

gkd49601

Figure 1. High resolution 5-methylcytosine map of the *BRCA1* 5′ region. Bisulfite modified DNA from peripheral blood lymphocytes (PBL), human foreskin fibroblasts (HFF), human mammary epithelial cells (HMEC) and the sporadic breast cancer cell lines MCF7 and UACC3199 were PCR amplified, cloned and sequenced. Clones from each cell type were analyzed to obtain a percent methylation of the 75 CpG dinucleotides in the *BRCA1* 5′ region located on the *y* axis. The *x* axis represents the nucleotide position relative to the *BRCA1* transcriptional start site (GenBank accession no. U37574).

gkd49602

Figure 2. Acetylation status of histones H3 and H4 in the *BRCA1* and *GAPDH* promoters in UACC3199, MCF7 and HMEC. Chromatin immunoprecipitations using acetyl-histone H3 and H4 antibodies were performed on UACC3199, MCF7 and HMEC. Following isolation of the acetyl-histone H3 and H4 enriched fraction of genomic DNA, the *BRCA1* or *GAPDH* promoters were PCR amplified. Presence of a PCR product indicates acetylation of the immunoprecipitated histone. Acetylation status of histone H3 (**A**) or histone H4 (**B**) for the *BRCA1* (top) and *GAPDH* (bottom) promoters in UACC3199, MCF7 and HMEC is shown. The different cell types analyzed are shown across the top and are grouped according to their incubation with acetyl-histone H3 or H4 antibody (+H3 Ab or +H4 Ab), no acetyl-histone antibody (–H3 Ab or –H4 Ab), or control sample. These experiments were performed three times each with similar results.

gkd49603

Figure 3. *Eco*RI chromatin accessibility assay of the *BRCA1* 5′ region. (**A**) A schematic showing the *in vivo Eco*RI and *in vitro Bam*HI restriction sites relative to the *BRCA1* transcription start site (bent arrow), the *BRCA1* probe, exons 1a and 1b, and the four possible cleavage products and their predicted sizes. (**B**) A Southern blot that shows each cell type and the resultant *in vivo Eco*RI digest products. The lanes, from left to right, are 0, 25, 75 and 225 U of *Eco*RI; the *in vitro* control *Eco*RI digest is shown in the fifth lane for each cell type. This experiment was performed three times with similar results.

gkd49604

Figure 4. *Sst*I chromatin accessibility assay of the *BRCA1* 5′ region. (**A**) A schematic showing the *in vivo Sst*I and *in vitro Bam*HI restriction sites relative to the *BRCA1* transcription start site (bent arrow), the *BRCA1* probe, exons 1a and 1b, and the four possible *in vivo Sst*I cleavage products and their predicted sizes. (**B**) A Southern blot that shows each cell type and the resultant *in vivo Sst*I digest products. The lanes, from left to right, are 0, 25, 75 and 225 U of *Sst*I; the *in vitro* control *Sst*I digest is shown in the fifth lane of each cell type. This experiment was performed three times with similar results.

gkd49605

Figure 5. Transcriptional repression of *BRCA1* by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the *BRCA1* 5′ regulatory region in sporadic breast cancer. Top, a schematic of the *BRCA1* 5′ flanking region. The *BRCA1* upstream region (hashed lines) contains a repetitive element (Alu) and extends to the beginning of the *BRCA1* 5′ regulatory region at –728 (black vertical line). The numbers represent the nucleotide position relative to the *BRCA1* transcription start site (bent arrow). *BRCA1*-positive normal breast cells (middle) are non-methylated, contain acetylated histones H3 and H4 (Ac) and have a protein accessible chromatin conformation (open ovals) in the *BRCA1* 5′ regulatory region. In contrast, the *BRCA1*-negative tumor cells (bottom) have an aberrantly methylated *BRCA15*′ regulatory region (meC), hypoacetylated histones H3 and H4 (deAc), and condensed chromatin (bricked ovals) coincident with *BRCA1* repression. Both normal and tumor cells have a methylated (meC) and chromatin condensed (bricked ovals) *BRCA1* upstream region.

AUTHOR - please check Figure legends carefully as they have been keyed in.

SUPPLEMENTARY MATERIAL

ArticleNucleic Acids ResearchNo2817ISSN 0305-1048gkd496http://www.nar.oupjournals.org/content/vol28/issue17/00001September2000

gkd496supp01

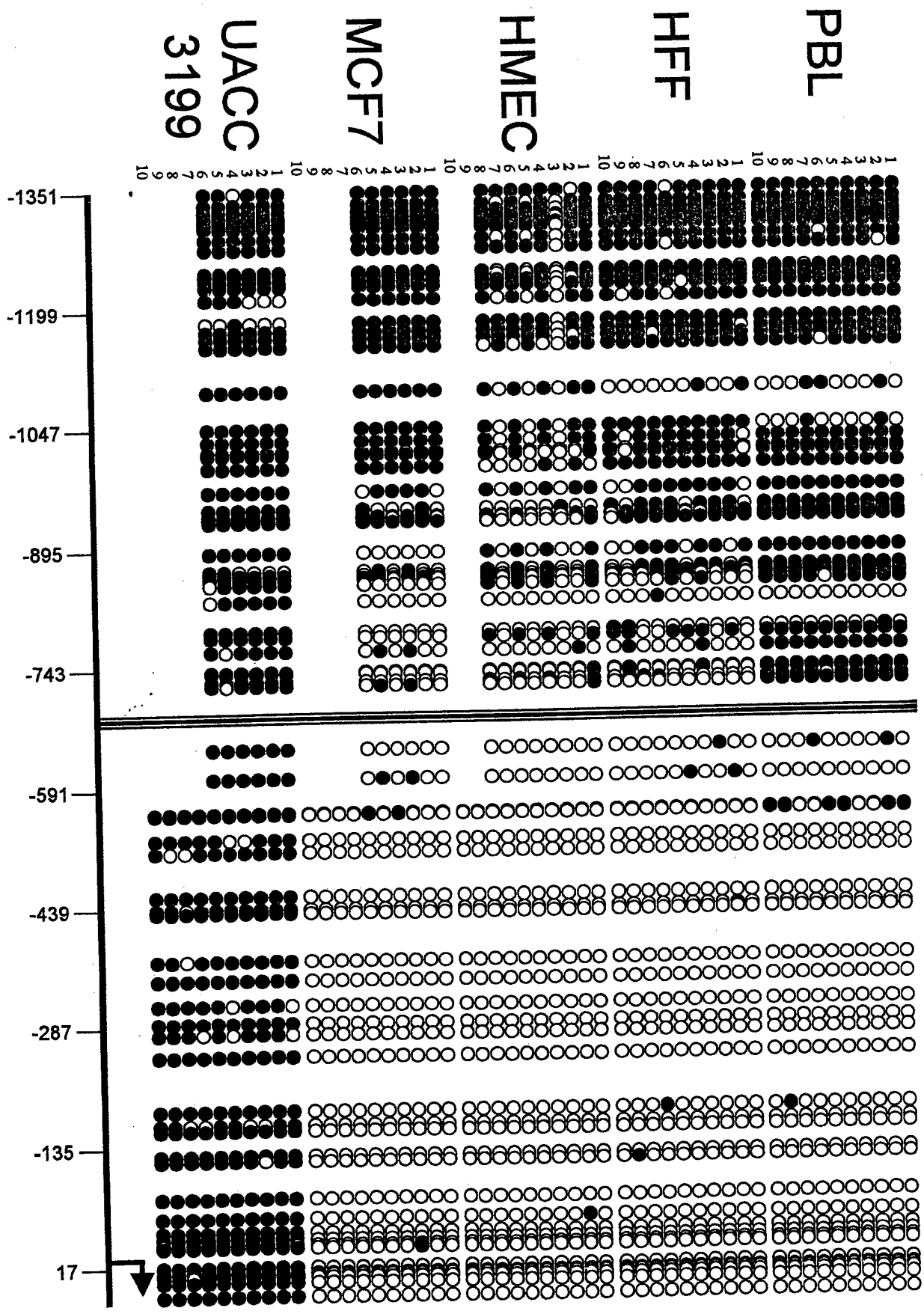
Figure S1. Methylation status of *p15* and *dCK* in UACC3199. Top, 57 CpG sites of the *p15* (GenBank accession no. S75756) CpG island were analyzed for methylation by sodium bisulfite genomic sequencing as described in the text using the following primers: primer 1 (nt 58–79), 5′-GGTTTTTTGGTTTAGTTGAAAA-3′: primer 2 (nt 698–725), 5′-AACITAAACTCCTATACAAATCTACAT-3′; primer 3 (nt 90–116), 5′-TGTIGGTTGGTTTTTTATTTTGTTAGA-3′; primer 4 (nt 608–634), 5′-AAACCCTAAAACCCCAACTACCTAAAT-3′. Bottom, 49 CpG sites of the ~~dCK~~ dCK (GenBank accession no. L07485) CpG island were analyzed using the following primers: primer 1 (nt 35–63), 5′-TTTGTTTATTTTTAATAGGTTTATTAGAG-3′; primer 2 (nt 577–603), 5′-AATCCCCTCAAAACTAACTAAAAAAAA-3′; primer 3 (nt 130–155), 5′-GGTTTTTGGGGTTTATTTTTTTTTTT-3′; primer 4 (nt 533–556), 5′-AAATAACCATTCCTTAATCTTATA-3′. These results show that these CpG islands have remained unmethylated in UACC3199, similar to normal tissue.

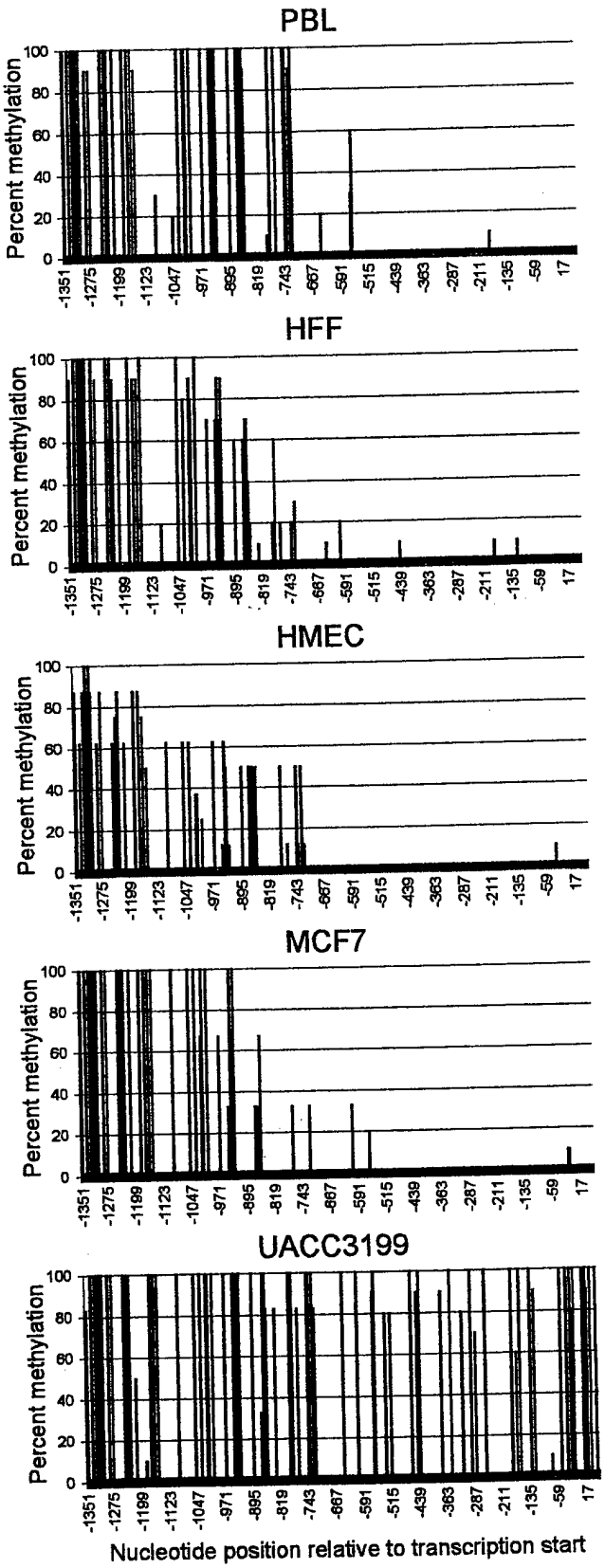
gkd496supp02

Figure S2. High resolution bisulfite sequencing data from HMEC and UACC3199. The sequence across the top of each electropherogram is the unmodified BRCA1 sequence from –568 to –553 relative to BRCA1 transcription start (GenBank accession no. U37574). HMEC is non-methylated at CpG –567 and –565 as each of these cytosines have been converted to thymidine ~~tymidine~~ in the bisulfite reaction (arrows). In contrast, UACC3199 is methylated at these CpG sites as the cytosines remain cytosine in the bisulfite reaction (arrows). All remaining non-methylated cytosines have been converted to thymidine ~~cytidine~~ in both HMEC and UACC3199.

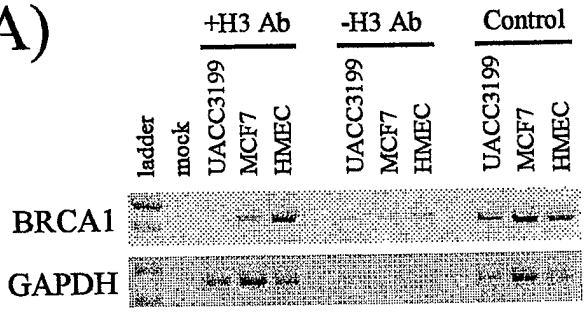
gkd496supp03

Figure S3. Shearing of protein-crosslinked DNA to small chromatin fragments. Genomic DNA was sonicated following formaldehyde cross-linking of proteins to genomic DNA to between 200 and 1000 bp in length. The gel shows that the bulk of the cross-linked genomic DNA is <1000 bp in both HMEC and UACC3199. This is, most likely, an overestimation of its true size considering protein bound DNA migrates slower than the naked DNA ladder as shown in this Figure.

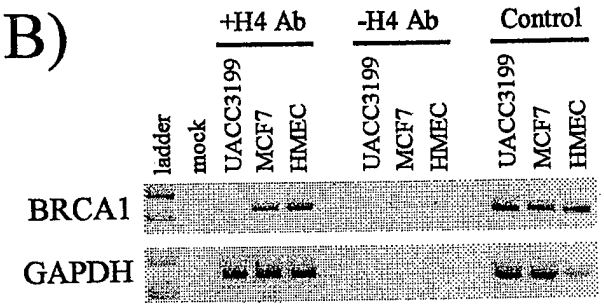

UACC 3199
MCF7
HMEC
HFF
PBL
-1351
-1199
-1047
-895
-743
-591
-439
-287
-135
17


PBL
HFF
HMEC
MCF7
UACC3199
Percent methylation
0
20
40
60
80
100
-1351
-1275
-1199
-1123
-1047
-971
-895
-819
-743
-667
-591
-515
-439
-363
-287
-211
-135
-59
17
Nucleotide position relative to transcription start

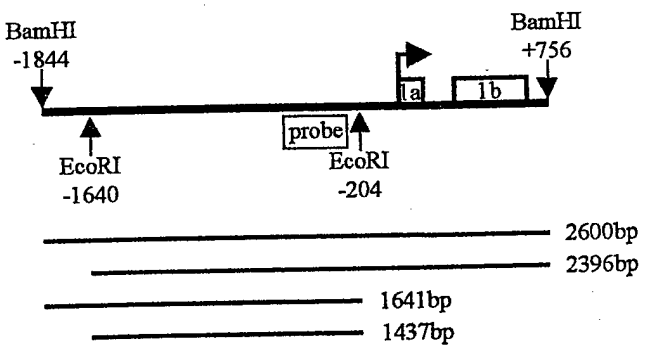
A)


+H3 Ab
-H3 Ab
Control
ladder
mock
UACC3199
MCF7
HMEC
UACC3199
MCF7
HMEC
UACC3199
MCF7
HMEC
BRCA1
GAPDH

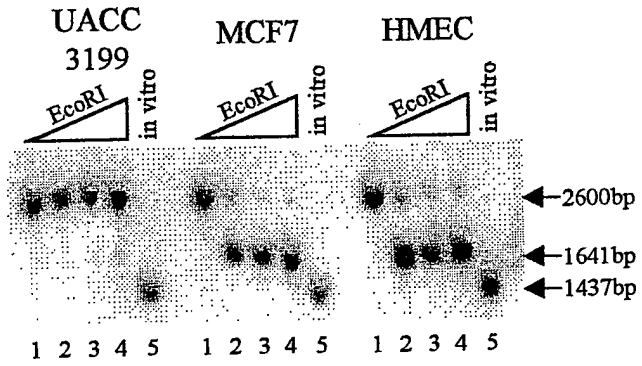
B)


+H4 Ab
-H4 Ab
Control
ladder
mock
UACC3199
MCF7
HMEC
UACC3199
MCF7
HMEC
UACC3199
MCF7
HMEC
BRCA1
GAPDH

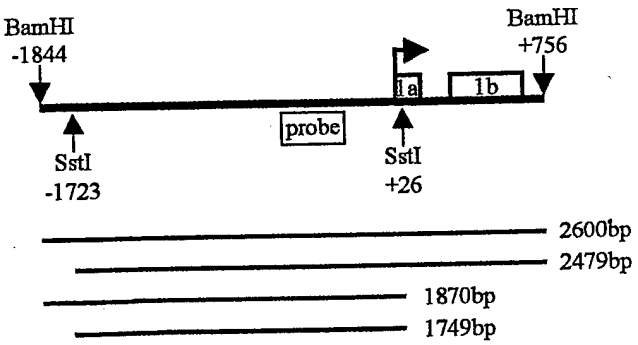
A)


BamHI
-1844
BamHI
+756
1a
1b
probe
EcoRI
-1640
EcoRI
-204
2600bp
2396bp
1641bp
1437bp

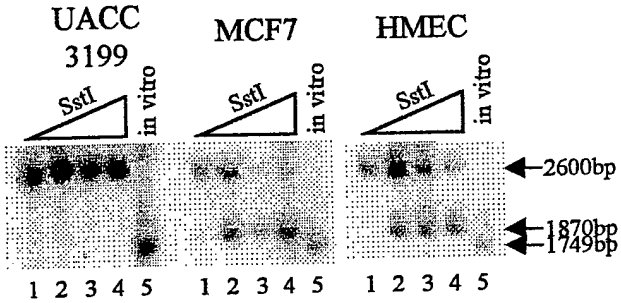
B)

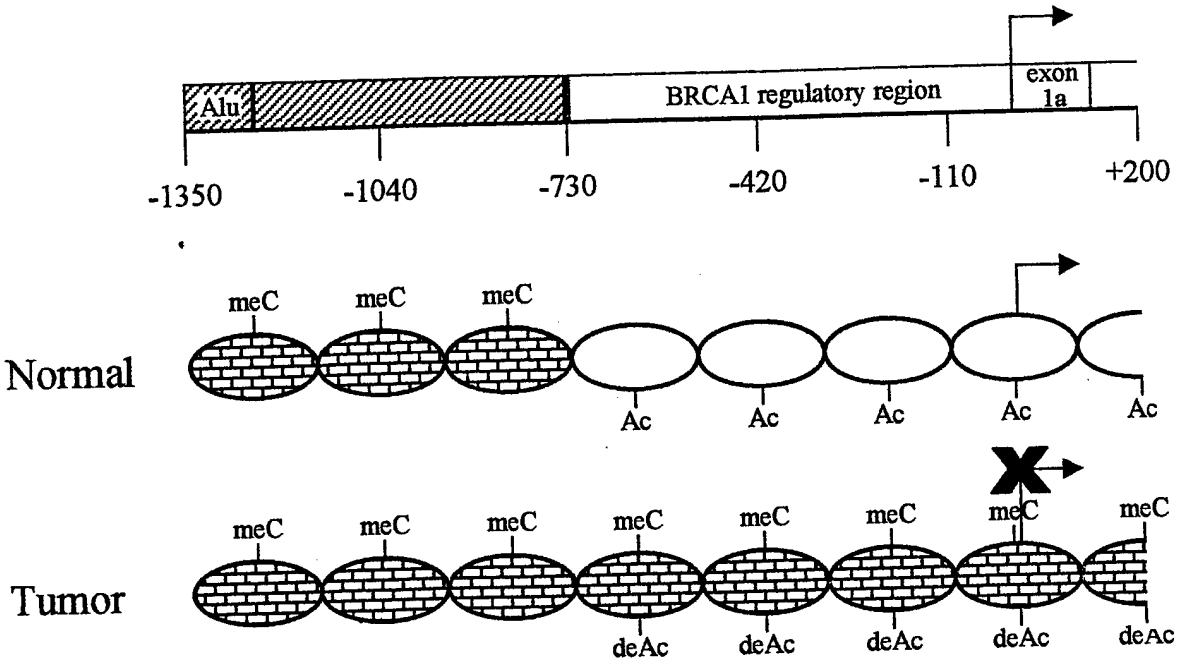

UACC
3199
MCF7
HMEC
EcoRI
in vitro
EcoRI
in vitro
EcoRI
in vitro
2600bp
1641bp
1437bp
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5

A)

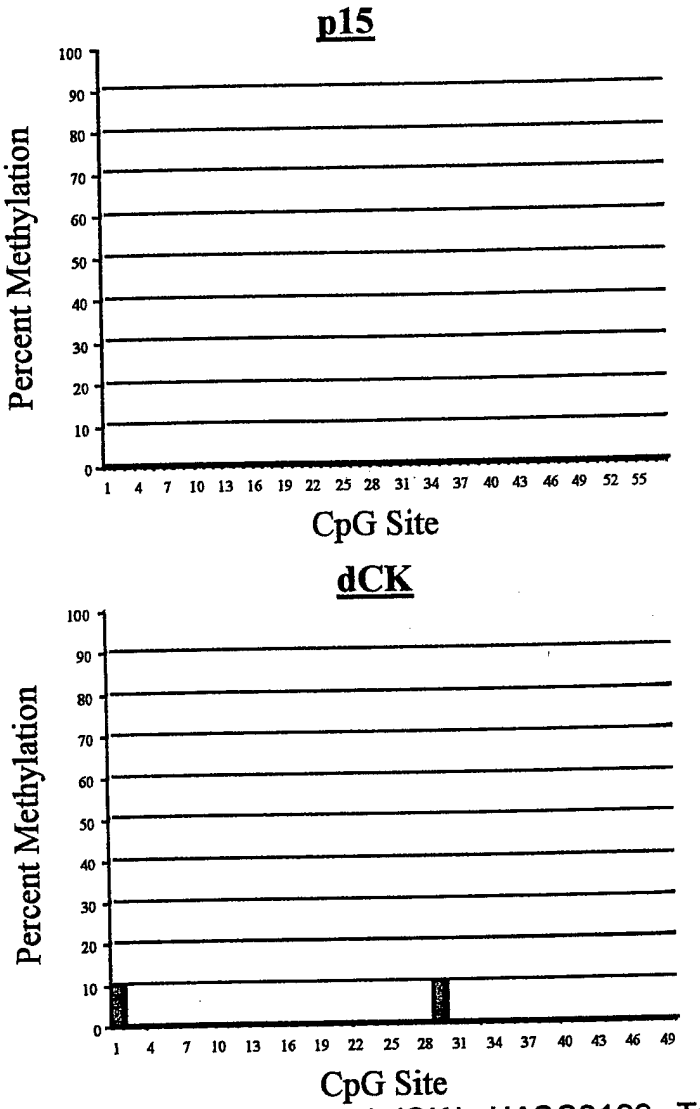

BamHI
-1844
BamHI
+756
1a
1b
probe
SstI
-1723
SstI
+26
2600bp
2479bp
1870bp
1749bp

B)


UACC
3199
MCF7
HMEC
SstI
in vitro
SstI
in vitro
SstI
in vitro
2600bp
1870bp
1749bp
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5

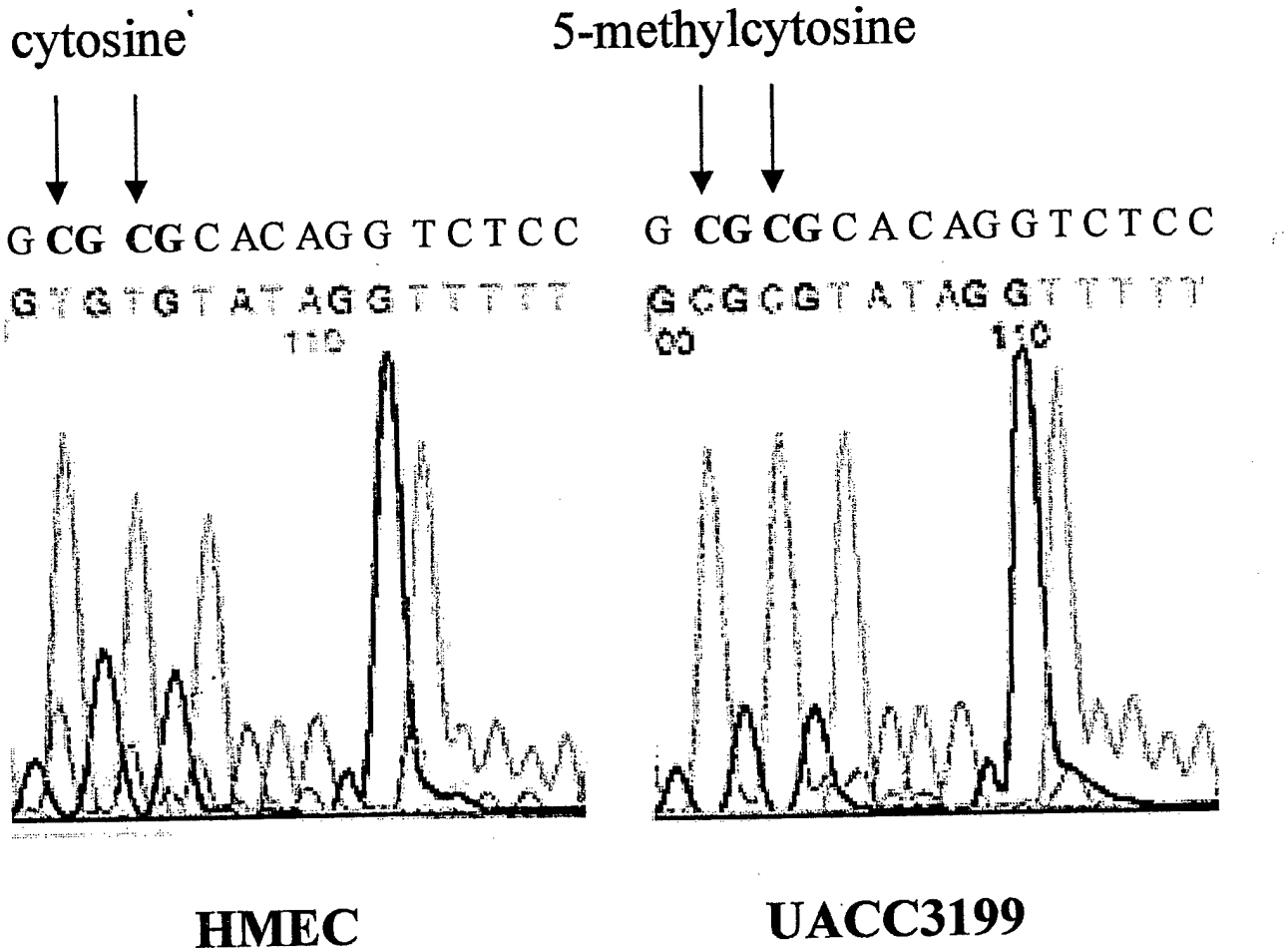

Alu
BRCA1 regulatory region
exon 1a
-1350
-1040
-730
-420
-110
+200
Normal
meC
meC
meC
Ac
Ac
Ac
Ac
Ac
Tumor
meC
meC
meC
meC
meC
meC
meC
meC
deAc
deAc
deAc
deAc
deAc

Supplemental Figure 1. Methylation Status of p15 and dCK in UACC3199.



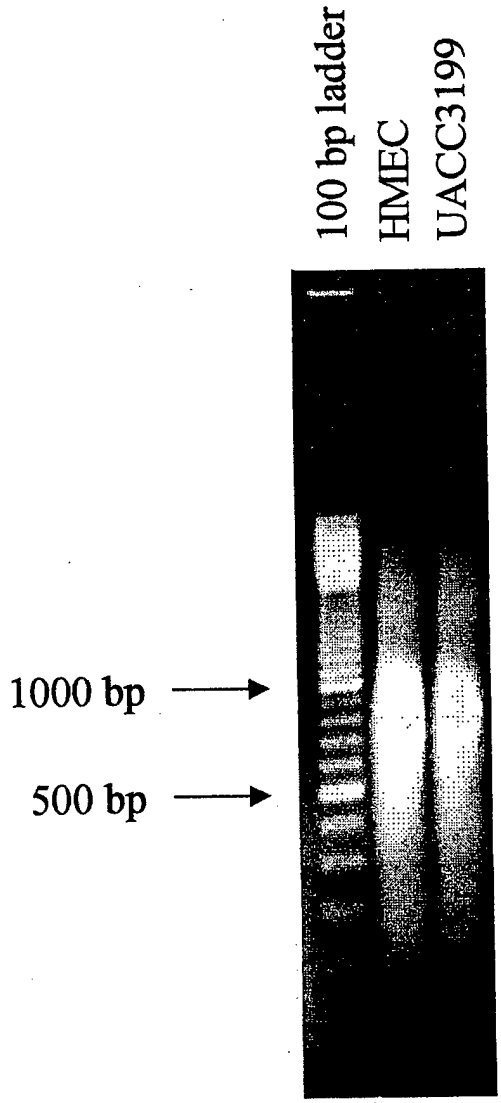
Supplemental Figure 2. Methylation status of *p15* and *dCK* in UACC3199. Top panel: 57 CpG sites of the *p15* (Genbank accession #S75756) CpG island were analyzed for methylation by sodium bisulfite genomic sequencing as described in the text using the following primers: primer 1 (nt 58-79), 5'-GGTTTTTTGGTTTAGTTGAAAA-3'; primer 2 (nt 698-725), 5'-AACITAAACTCCTATACAAATCTACAT-3'; primer 3 (nt 90-116), 5'-TGTIGGTTGGTTTTTTATTTTGTTAGA-3'; primer 4 (nt 608-634), 5'-AAACCCTAAAAC CCCAACTACCTAAAT-3'. Bottom panel: 49 CpG sites of the *dCK* (Genbank accession #L07485) CpG island were analyzed using the following primers: primer 1 (nt 35-63), 5'-TTTGTTTATTTTTAATAGGTTTATT AGAG-3'; primer 2 (nt 577-603), 5'-AATCCCCTCAAAACTAACTAAAAAAAA-3'; primer 3 (nt 130-155), 5'-GGT TTTTGGGGTTTATTTTTTTTTTT-3'; primer 4 (nt 533-556), 5'-AAATAACCATTCCTTAATCTTATA-3'. These results show that these CpG islands have remained unmethylated in UACC3199, similar to normal tissue.

Supplemental Figure 2. High resolution bisulfite sequencing data from HMEC and UACC3199.



Supplemental Figure 2. High resolution bisulfite sequencing data from HMEC and UACC3199. The sequence across the top of each electroferogram is the unmodified BRCA1 sequence from -568 to -553 relative to BRCA1 transcription start (Genbank accession #U37574). HMEC is nonmethylated at CpG -567 and -565 as each of these cytosines have been converted to tymidine in the bisulfite reaction (arrows). In contrast, UACC3199 is methylated at these CpG sites as the cytosines remain cytosine in the bisulfite reaction (arrows). All remaining nonmethylated cytosines have been converted to tymidine in both HMEC and UACC3199.

Supplemental Figure 3. Shearing of protein-crosslinked DNA to small chromatin fragments.



Supplemental Figure 3. Shearing of protein-crosslinked DNA to small chromatin fragments. Genomic DNA was sonicated following formaldehyde cross-linking of proteins to genomic DNA to between 200 bp and 1000 bp in length. The gel shows that the bulk of the cross-linked genomic DNA is less than 1000 bp in both HMEC and UACC3199. This is, likely, an overestimation of its true size considering protein bound DNA migrates slower than the naked DNA ladder as shown in the figure.